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Molin et al.

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[54] **PRODUCTION IN *ESCHERICHIA COLI* OF EXTRACELLULAR SERRATIA SPP. HYDROLASES**

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[21] Appl. No.: **476,960**

[22] Filed: **Feb. 7, 1990**

Related U.S. Application Data

[62] Division of Ser. No. 372,679, Jun. 28, 1989, abandoned, which is a division of Ser. No. 20,943, Jan. 8, 1987, abandoned.

[30] **Foreign Application Priority Data**

May 10, 1985 [DK] Denmark 2100/85
Dec. 23, 1985 [DK] Denmark 6060/85

[51] **Int. Cl.**[5] **C12N 9/20**; C12N 9/22; C12N 15/55

[52] **U.S. Cl.** **435/198**; 435/199; 536/27

[58] **Field of Search** 435/69.1, 72, 252.33

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Attorney, Agent, or Firm—Iver P. Cooper

[57] **ABSTRACT**

Extracellular Serratia spp. enzymes have been found to be excreted by another gram-negative organism harbouring a plasmid carrying DNA from Serratia spp. encoding the enzymes. This organism, e.g. *E. coli,* is therefore employed to produce the enzymes, specific examples of Serratia spp. enzymes produced are a nuclease, a lipase and a phosopholipase. The nuclease may be employed to remove nucleic acids from a biological material.

10 Claims, 13 Drawing Sheets

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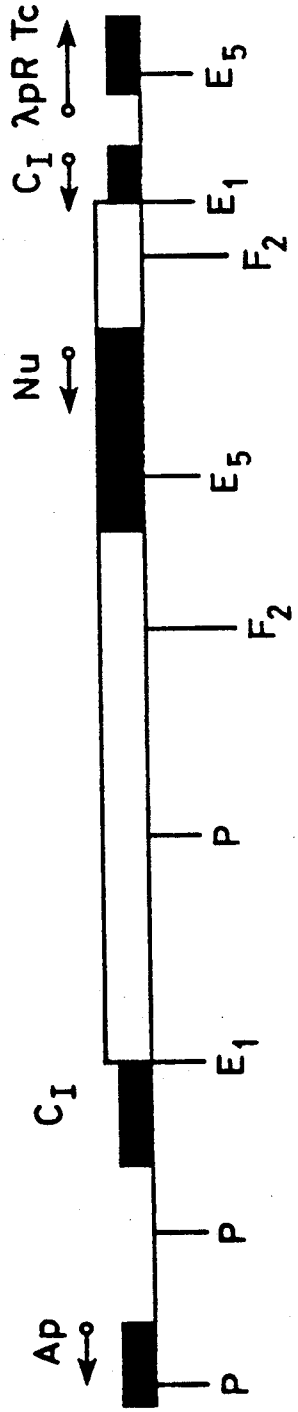


pNU121-*nuc*$^+$

Fig. 1

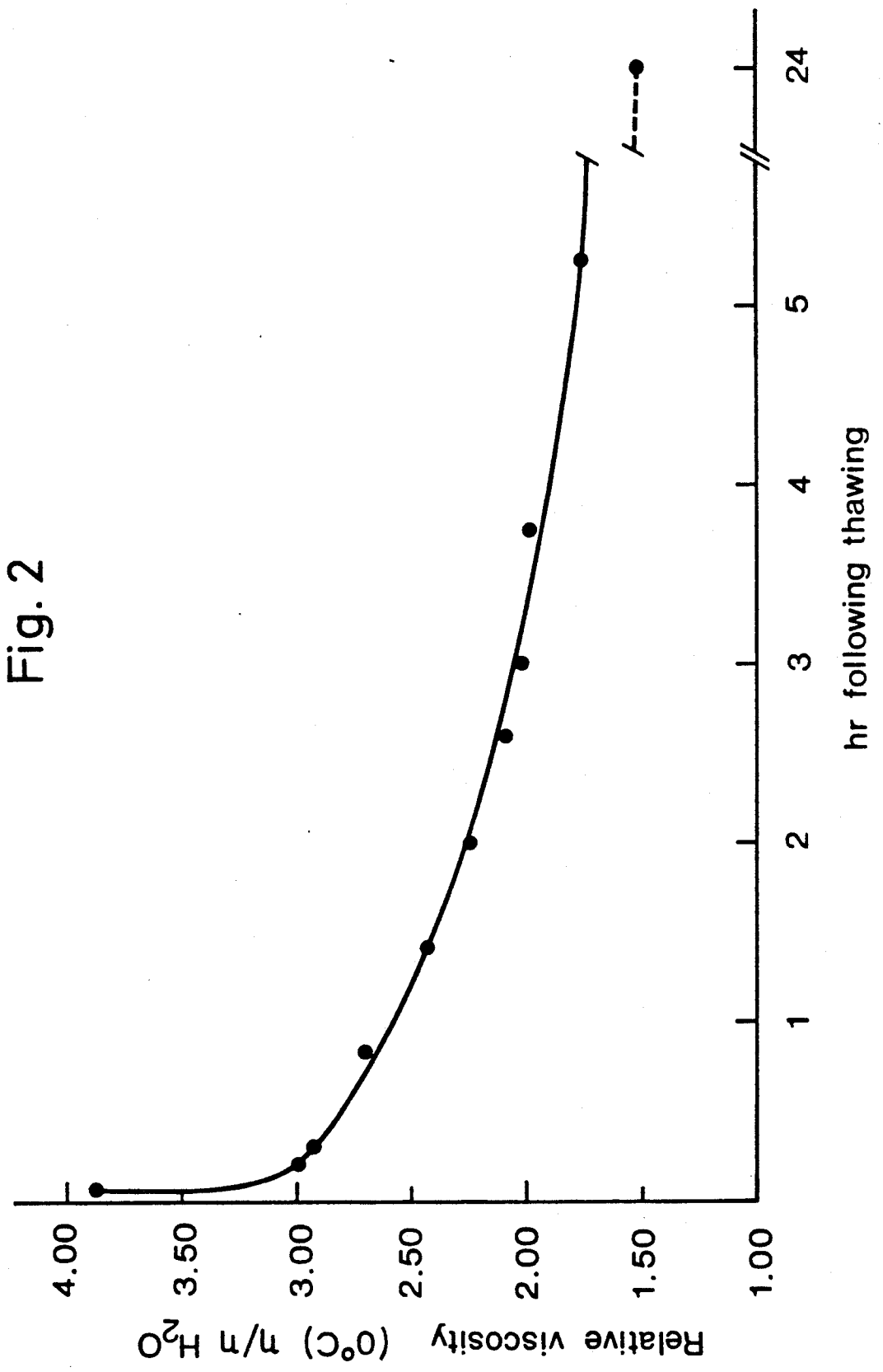
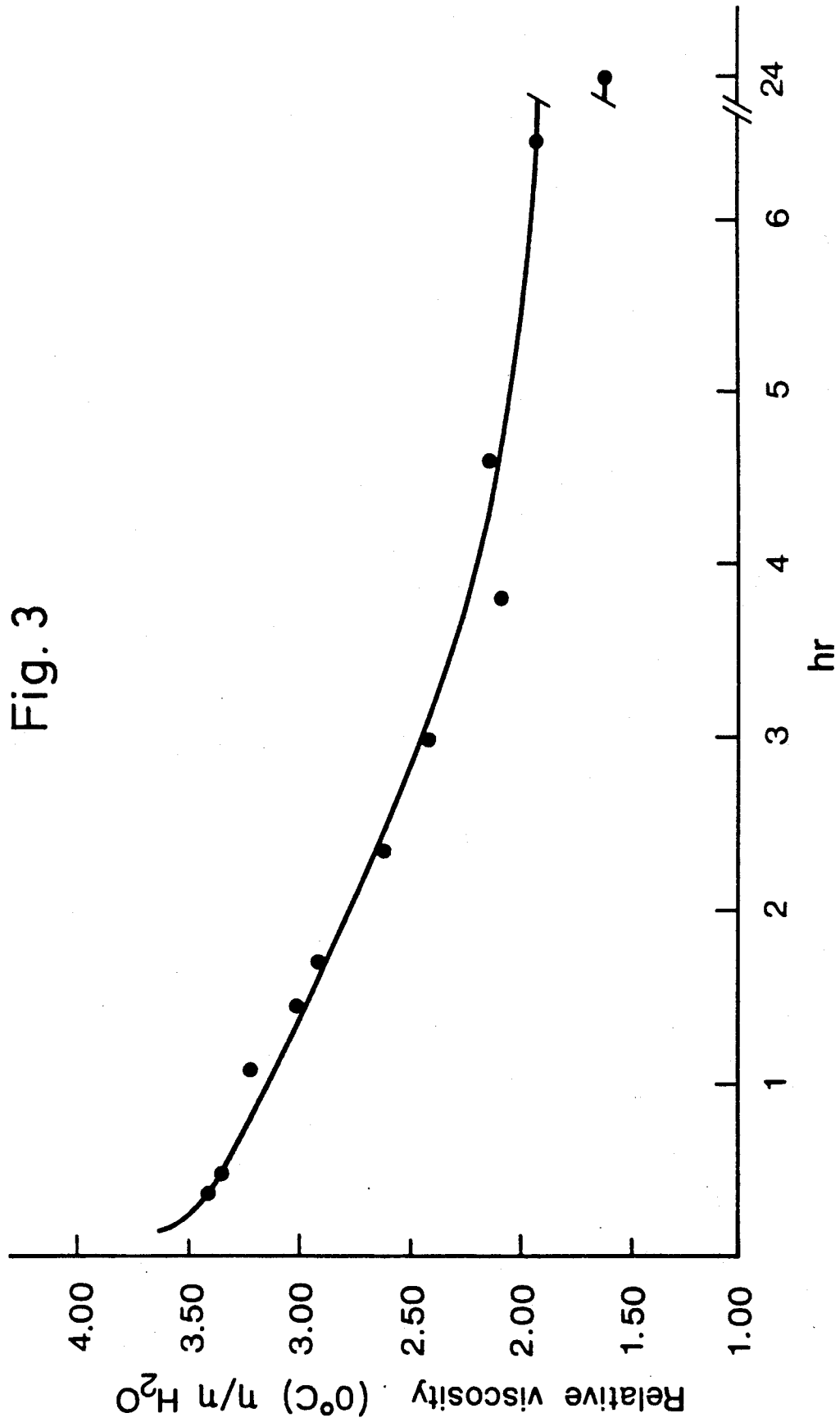

Relative viscosity (0°C) η/η H2O
4.00
3.50
3.00
2.50
2.00
1.50
1.00
1
2
3
4
5
24
hr following thawing

Fig. 2


Relative viscosity (0°C) η/η H2O
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3.50
3.00
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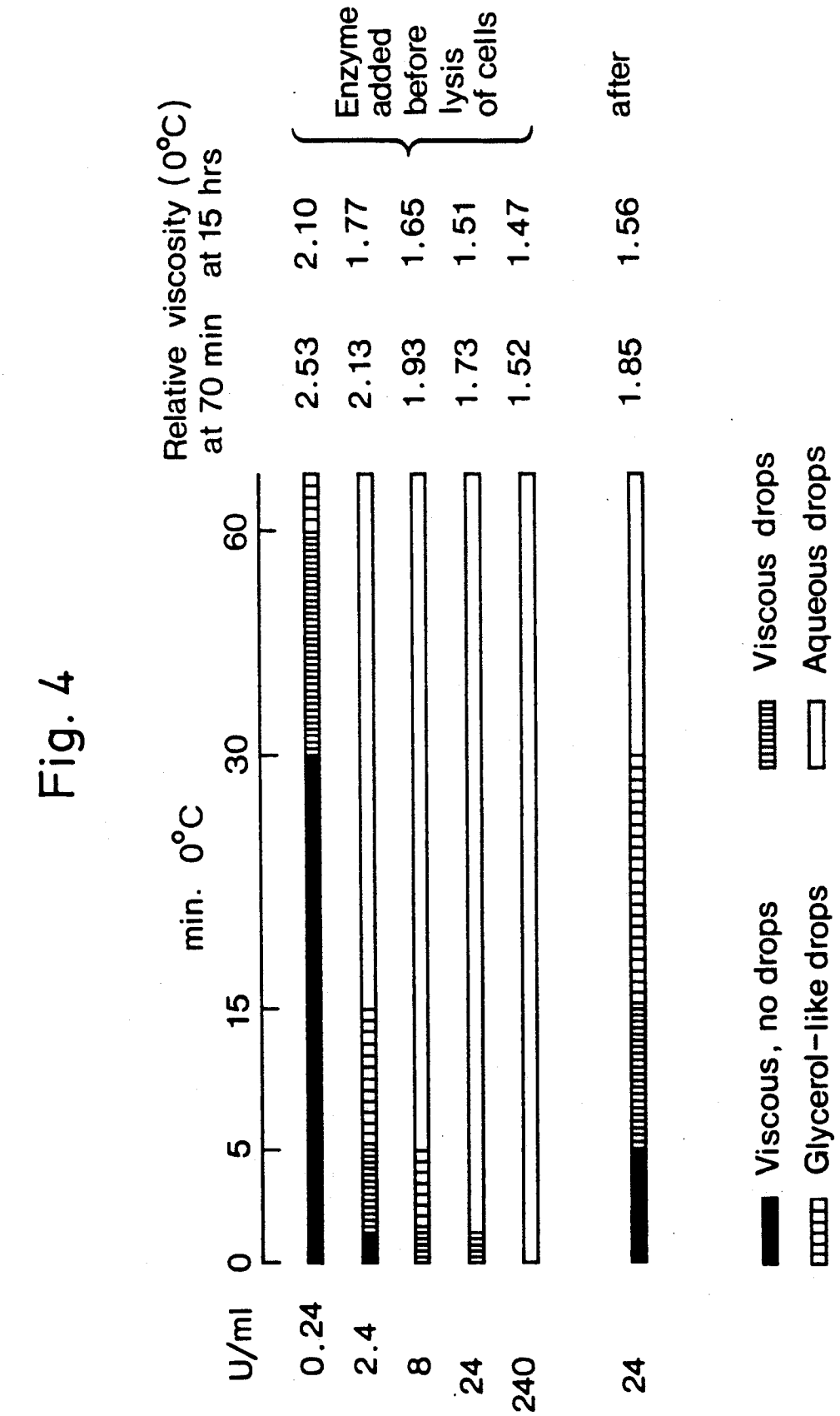
1.50
1.00
1
2
3
4
5
6
24
hr

Fig. 3

Fig. 4


Relative viscosity (0°C)
at 70 min at 15 hrs
min. 0°C
0
5
15
30
60
U/ml
0.24
2.4
8
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240
24
2.53
2.13
1.93
1.73
1.52
1.85
2.10
1.77
1.65
1.51
1.47
1.56
Enzyme added before lysis of cells
after
Viscous, no drops
Glycerol-like drops
Viscous drops
Aqueous drops

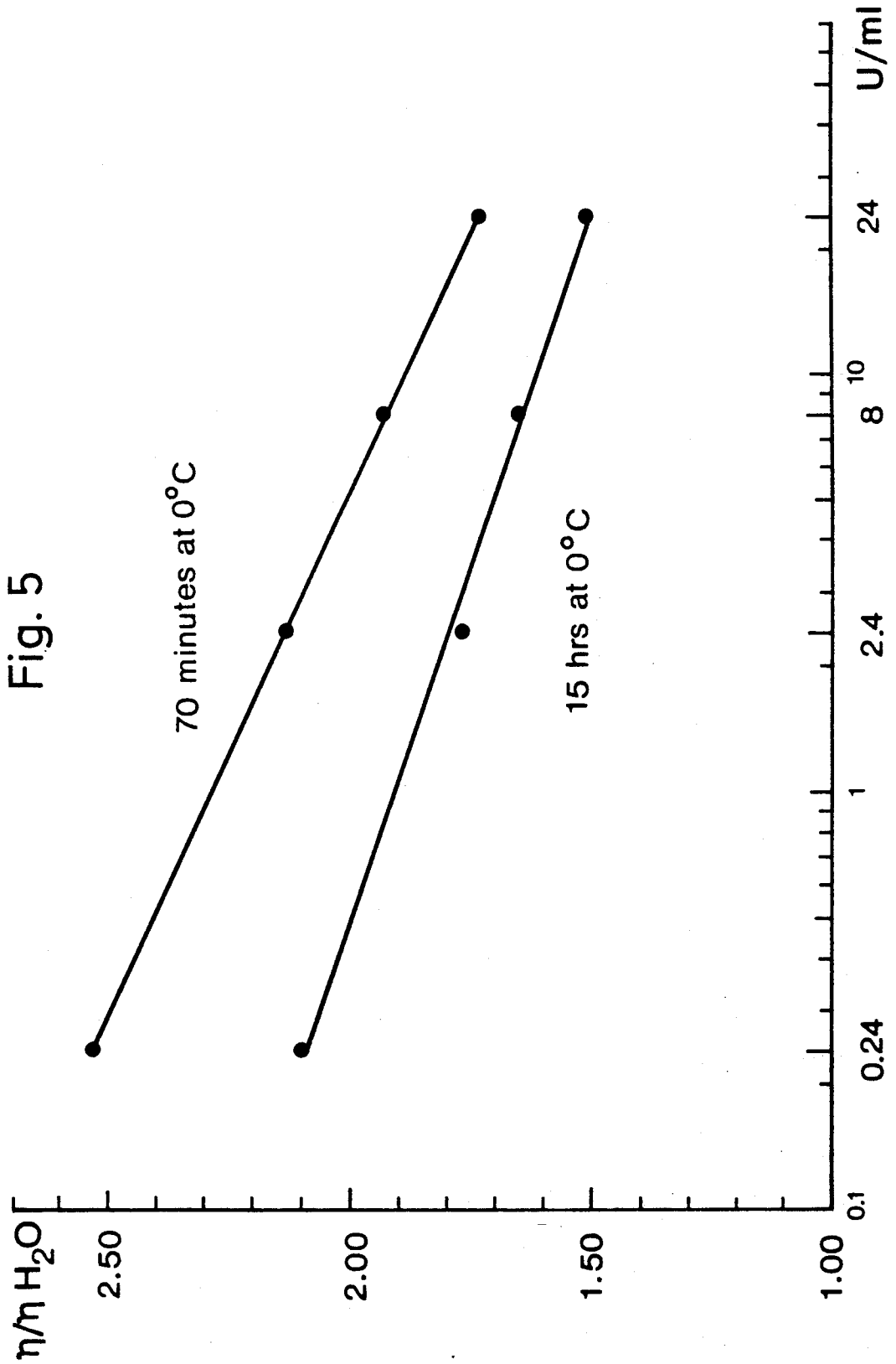
η/η H2O
2.50
2.00
1.50
1.00
0.1
0.24
1
2.4
8
10
24
U/ml
70 minutes at 0°C
15 hrs at 0°C

Fig. 5

CORRESPONDING TO LINE

2 3 4 5 6 IN FIG. 4

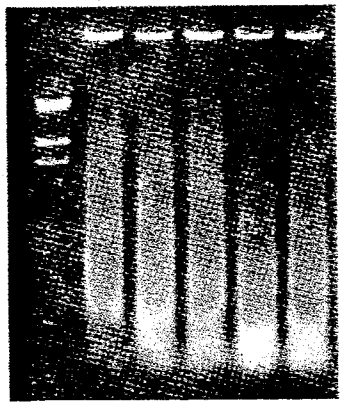

Fig. 6

```
1
GCCCCGCTGAAAGTCGTCTTGAAGGTGCTGCTGTCTTCGGTGGACAAGATAGTCAAAGTGCAGATCGCCCAACAAGGTCGAGCAGGAAATCGCCCAGATCG
CGGGGCGACTTTCAGCAGAACTTCCACGACGACAGAAGCCACCTGTTCTATCAGTTTCAGCGGTTGTTCCAGCTCGTCCTTTAGCGGGTCTAGC

101
AACAGCGGTGGTGCGCCGCCGGCGGTCCGGTATCACCGGCGATCTGGCGCAGATGAGCGCATTAGGTTCGCCGCGACAGGTCAACGACCTGCTGGATAATC
TTGTCGCCACCACGCGGGCGGCCGCCGCGCCATAGTGGCCGCTACTGCGTAATGCCAAGCCGCGTGCCAGTTGCTGGAGGACCTATTAG

201
CCCCCTTTGAAACGGCGTCTGTTGACGCGCTTTATTTCCGCCGCATTTCACGTGCGGCGTGGCTGTACCATGACTGACACATTCACACAACATGAATATGTT
GGGGGAAACTTTGCCGCAGACAACCTGCCGAAATAAAGGCGGCGTAAAGTGCACGCCGCACCGCATGCTACTGACTGTGTAAGTGTTGTACTTATACAA

301
GCATTGTTGTATTCGTTTCACTGCGATAAGTTTAATTTACTGTAAATATATATACAGTACTTTTTTTTAACTTATTGAGGATATGAATATGCGCTTTAACAAC
CGTAACAACATAAGCAAAGTGACGCTATTCAAATTAAATGACATTTATATATGTCATGAAAAAAATTGAATAACTCCTATACTTATAGCGCGAAATTGTTG

401
AAGATGTTGGCCTTGGTCGCCCTGCTGTTCGCCGCACAGGCGTCGCCGACAGCTCGAATCCATCGACAACTGCGCGGTCGGCTGCCCGACCGCGCGCCA
TTCTACAACCGGAACCAGCGGGACGACAAGCGGCGTGTCCGCAGCCGGCTTGAGCTGTTGAGCGCGCCAGCCGACGGGCTGGCCGCCGT

501
GCAGCAACGTGTCTATCGTGCGTCATGCTTATACGTTGAACAACAACAGCACCACCAAGTTCGCCAACTGGGTGCCTTATTCACATCACCAAAGACACACC
CGTCGTTGCACAGATAGCACGCAGTACGAATATGCAACTTGTTGTTCTCGTGGTGGTTCAAGCGGTTGACCCACCGAATAGTGTAGTGGTTTCTGTGTGG

601
GGCCACGCGGCAAGACGCGCAACTGGAAAACCGATCCGGCCTCAACCCGGCGGACACGTTGGCGCCCGCCGATTACACTGGCGCCAAGCGGCGCCTGAAG
CCGGTGCGCCGTTCTGCGCGTTGACCTTTTGGCTAGGCCGCGACTTGGGCCGCCTGTGCAACCGCGGCGGCGCTAATGTGACCGCGGTTGCGCCCGCGACTTC
```

Fig. 7a.

```
701   .    .    .    .    750  .    .    .    .    800
GTCGATCGCGGTCATCAGGCGCGCTGGCCTCGCTGGCGGGCGTCTCCGACTGGGAATCGCTGCCTGAATTACCTGTCCAACATCACGCCGCAAAAGTCCGATC
CAGCTAGCGCCAGTAGTCCGCGCGACCGCGAGAGGCTGACCCTTAGCGACTTAATGGACAGGTTGTAGTGCGGCGTTTTCAGGCTAG

801   .    .    .    .    850  .    .    .    .    900
TTAACCAGGGCGCCTGGCGCGCGCTGGAAGATCAGGAACGCAAGCTGATCGATCGCGCCGATATCTCCTCGGTCTATACCGTGACCGCGCCCGCTGTATGA
AATTGGTCCCGCGGACCCGCGCGCCGACCTTCTAGTCCTTGCGTTCGACTAGCTAGCGGCCGGCTATAGAGGAGCCAGATATGGCACTGCGCCCGGGCACATACT

901   .    .    .    .    950  .    .    .    .    1000
ACGCGATATGGCCAAACTGCCGGGCACCCAGAAAGCGCACACCATCCCCAGCGCCTACTGCAAGGTGATTTTCATCAACAACAGCCCGCGCGGTGAACCAC
TGCGCTATACCGTTTGACGGCCCGTGGTCTTTCGCGTGTGGTAGGGGTCGCGCGATGACCTTCCACTAAAAGTAGTTGTTGTCGCGCCCCACTTGGTG

1001  .    .    .    .    1050 .    .    .    .    1100
TATGCCGCTTTCCTGTTCGATCAGAACACGCCGAAGGGGCGCCGATTTCTGCCAATTCGCGCGTGACGGTGGACGAGATGGAAACGGCACCGGGCCTGATCA
ATACGGCGAAAGGACAAGCTAGTCTTGTGCGGCTTCCCGCGCGCTAAAGACGGTTAAGGCCGCACTGCCACACCTGCTCTAGCTCTTTGCGTGGCCCGGACTAGT

1101  .    .    .    .    1150 .    .    .    .    1200
TCTGGGCCCGGTCTGCCCGGACGACGTGCAGGCTTCGCTGAAGAGCAAACCGCGCGTCCTGCCGGAGTTGATGGGCTGCAAAAACTGACGAAAACCGCCGAAG
AGACCCGGGCCAGACGGGCCTGCTGCACGTCCGAAGCGGACTTCTCGTTTGGCCGCAGGACGGCCTCAACTACCCGACGTTTTTGACTGCTTTTTGGCGGCTTC

1201  .    .    .    .    1250 .    .    .    .    1294
CGGGTTTATTTTTTCACGCGGGCGCCGGCGATTATCCCGTCGCGCCTTTTGCGCGGCGGCCAACTCACGCTGACGGGGTGAGGCTACCGGGGCCC
GCCCAAATAAAAAAGTGCGCCCGCGGCCGCCCTAATAGGGCAGCGCGCGAAAACGCCGCCGCCGGTTGAGTGCGACTGCCCCACTCCGATGGCCCCGG
```

Fig. 7b.

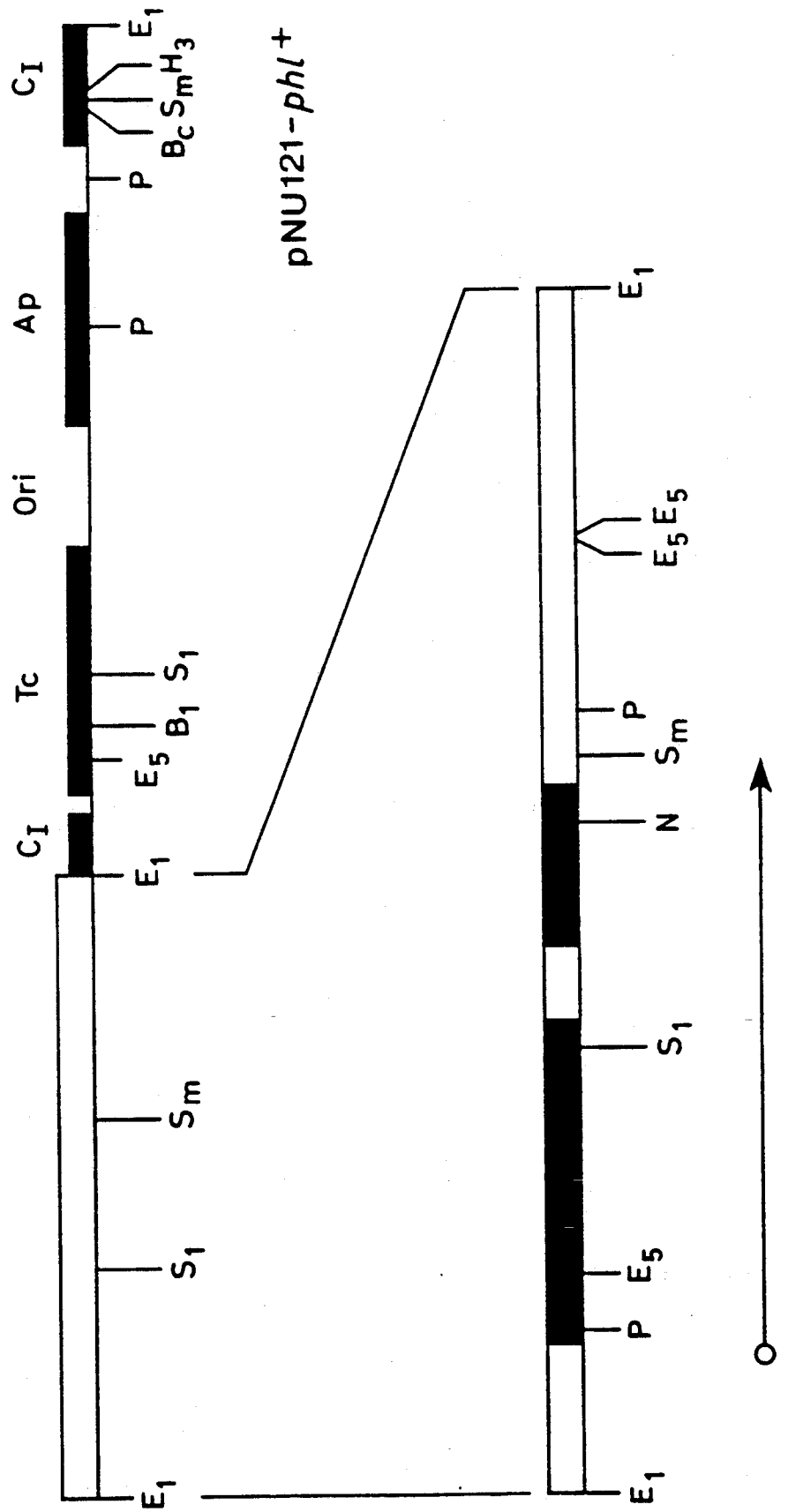
pNU121-phl+
CI
E1
BcSmH3
P
Ap
P
Ori
Tc
S1
B1
E5
CI
E1
Sm
S1
E1
E1
E5E5
P
Sm
N
S1
E5
P
E1

Fig. 8

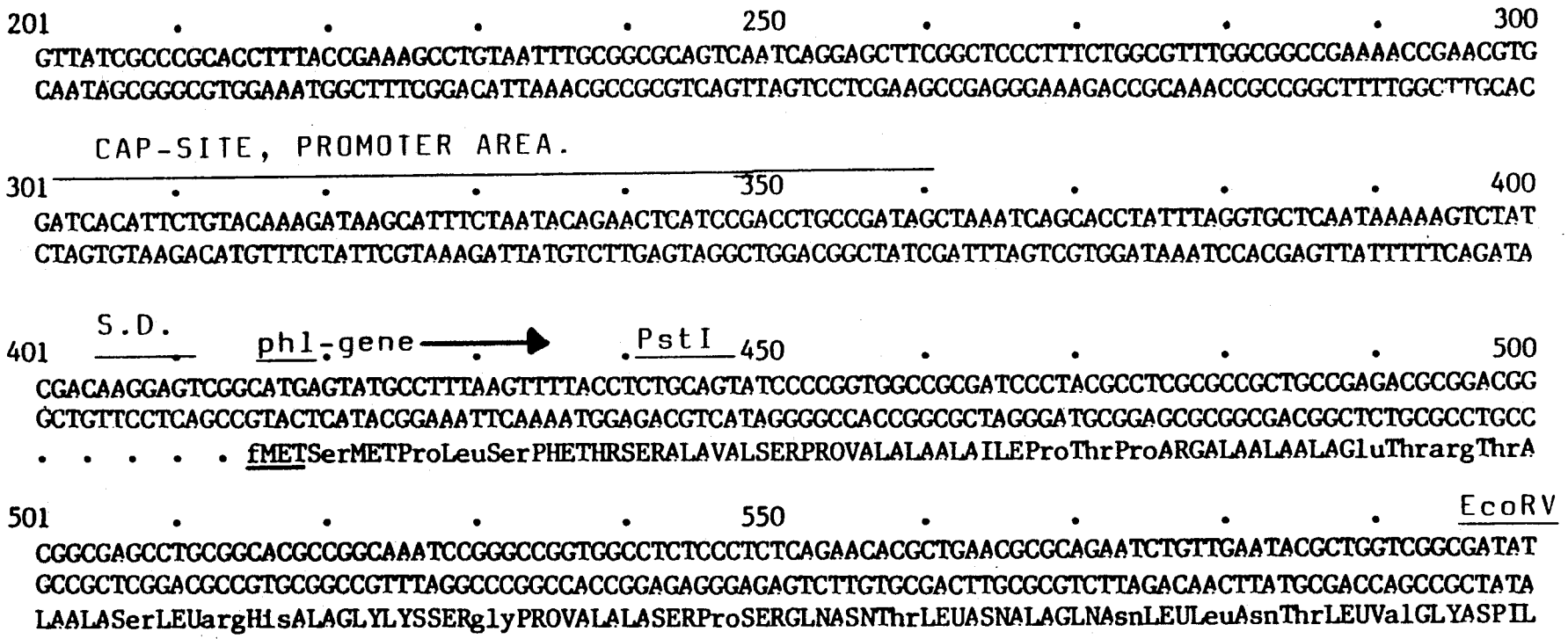

Fig. 9a.

```
601        .         .         .         650         .         .         .         700
CTCAGCGCGCGGCACCGACGGCGGCGGCAGCGCCCGGGCGGTGACCGCAGGAGGGGGATTATGCGTTGGCGCTGTTGGCCAAGGACGTT
GAGTCGCCCGCCGTGGCTGCCGCCGCCGCCGTCGCTTAGCGTCCTCCCCCCTAATACGCAACCGCGACAACCGGTTCCTGCAA
ESerALAALAALAPROThrALAALAALAALAPROGLYVALThrargglyGLNGlnSerGLNGluglyASPTYRALALeuALALEULeuALALysASPVAL

701        .         .         .         750         .         .         .         800
TACTCACTCAATGGCCAGGGCGCCCGCCCGGTTCAACCCGCCTGAGCGACAGCGCGCTGCTCGGTTTCGGCATCGATCCCCGCCAGCCTGCACGACCGCGGCCA
ATGAGTGAGTTACCGGTCCCGCGGCGGCCCAAGTTGGCGGACTCGCTGTCGCGCGACGAGCCAAAGCCGTAGCTAGGGCGGGTCGGACGTGCTGCGCCCGT
TYRSerLeuAsnGLYGLNGLYALAALAglyPHEASNARGLEUSerASPSerALALEULeuGLYPHEGLYILEASPProALASerLEUHisASPALAGLYS

801        .         .         .         850         .         .         .         900
GCGGTTTCCAGGCTGGGATTTACAGCAACGACAAACAGTATGTGTTGGCGTTCGCCGGCACCAACGACTGGCGCGATTGGCTGAGCAACGTGCGGCAGGC
CGCCAAAGGTCCGACCCTAAATGTCGTTGCTGTTTGTCATACACAACCGCAAGCGGCCGTGGTTGCTGACCGCGCTAACCGACTCGTTGCACGCCGTCCG
erGLYPHEGLNALAglyILETYRSerASNASPLYSGLNTYRVALLeuALAPHEALAGLYTHRASNASPTRPARGASPTRPLEUSerASNVALargGLNAL
```

Fig. 9b.

```
901                 .                 .                 .                 .                950                 .                 .                 .                 .               1000
GACGGGCTATGACGATGTGCAGTACAATCAGGCGGTTGCCGCTGCCAAAAGCCGCCAAGGCGGCCTTCGGCGATGCGCTGGTGATCGCCGGCCATTCGCT
CTGCCCGATACTGCTACACGTCATGTTAGTCCGCCAACGGCGACGGTTTTCGGCGGTTCCGCCGGAAGCCGCTACGCGACCACTAGCGGCCGGTAAGCGA
AThrGLYTYRASPASPVALGLNTYRAsnGLNALAVALALAALAALALYSSerARGGlnGLYGLYLeuargargCYSALAGLYASPARGargProPHEALA

1001                .                 .                 .                 .               1050                .                 .                 .                 .               1100
TGGCGGTGGTCTGGCGGCCACCGCCGCGCTGGCGACCGGCACCGTCGCGGTCACCTTCAACGCGGCCGGGGTCTCGGATTACACCCTGAATCGCCTGGGC
ACCGCCACCAGACCGCCGGTGGCGGCGCGACCGCTGGCCGTGGCAGCGCCAGTGGAAGTTGCGCCGGCCCCAGAGCCTAATGTGGGACTTAGCGGACCCG
TRPargTRPSERGLYGLYHisARGARGALAGLYASPargHisARGARGGLYHisLeuGlnARGGLYargGLYLeuglyLeuHisProGLUSerProglyH

1101                .                 .                 .                 .               1150                .                 .                 .  SalI           1200
ATCGATCCGGCGGCAGCGAAGAAAGATGCCGAAGCCGGCGGCATTCGCCGTACAGCGAGCAATATGACATGCTGACCAGCACCCAGGAGTCGACCTCGCT
TAGCTAGGCCGCCGTCGCTTCTTTCTACGGCTTCGGCCGCCGTAAGCGGCATGTCGCTCGTTATACTGTACGACTGGTCGTGGGTCCTCAGCTGGAGCGA
ISargSERGLYGLYSerGLUGLUargCYSargSerargargHISSerPROTYRSerGluGlnTYRASPMETLEUTHRSerTHRGLNGluSerTHRSerLE

1201                .                 .                 .                 .               1250                .                 .                 .                 .               1300
GATCCCGGATGCCATCGGCCACAACATCACCCTGGCCAACAACGATACCCTGACCGGCATCGATGACTGGCGGCCGAGCAAACATCTGGATCGCAGCCTG
CTAGGGCCTACGGTAGCCGGTGTTGTAGTGGGACCGGTTGTTGCTATGGGACTGGCCGTAGCTACTGACCGCCGGCTCGTTTGTAGACCTAGCGTCGGAC
UILEPROASPALAILEGLYHisASNILETHRLEUALAASNASNASPTHRLEUTHRGLYILEASPASPTRPargPROSerLYSHISLEUASPARGSerLEU
```

Fig. 9c.

```
1301  .    .    .    1350   phl-stop  .    .    .    1400
ACGGCGCACGGCATCGACAAGGTGATAAGCTCGATGGCGGAACAAAAGCCGTGGGAGGCGAAGGCCAATGCCTGAAGGGCGTCGCTTGCGGCGGGCGCTG
TGCCGCGTGCCGTAGCTGTTCCACTATTCGAGCTACCGCCTTGTTTTCGGCACCCTCCGCTTCCGGTTACGGACTTCCCCGCAGCGAACGCCCGCCCCGAC
ThrALAHisGLYILEASPLysVALileSerSerMETALAGLUGlnLysPROTRPGluALALysALAAsnALA.

1401  .    .    .    1450   .    .    .    .    1500
GCGATAGCCTTACTGGCGCTGGTCGGGTGACCGGTTTACTGATGATGGCTAAGGAGCAACAGATGGGGCAGGAGATTTCGCGTTTGACGGCCACAGCG
CGCTATCGGAATGACCGCGACCAGCGCCACTGGCCAAATGACTACTACCGATTCCTCGTTGTCTACCCCGTCCTCTAAAGCGGCAAACTGCCGGTGTCGC

1501  .    .    .    1550   SplI .    .    .    .    1600
AATCTGGCGCTGGCTCAGGCGGTGGCGCGTGGCGATACGCAGGGTATCCATGCGCAGGCCACGGCAGGATCGCTTGCCGCGAACGGGCGCAGATCGGCAGGTCA
TTAGACCGCGACCGAGTCCGCCACCGCGCACCGCTATGCGTCCCATAGGTACGCGTCCGGTGCGTCCTAGCGAACGCGCTTGCCCCGCTAGCCGTCCAGT
```

Fig. 9d.

PRODUCTION IN *ESCHERICHIA COLI* OF EXTRACELLULAR SERRATIA SPP. HYDROLASES

This is a divisional of U.S. application Ser. No. 07/372,679, now abandoned, filed Jun. 28, 1989, which is a divisional of U.S. application Ser. No. 07/020,943, filed Jan. 8, 1987, now abandoned.

The present invention relates to a method of producing bacterial enzymes and to hybrid plasmids and microorganisms useful in the method. The invention further relates to the use of one of the enzymes, nuclease, for removing nucleic acids from a biological material, as well as to a regulatory region useful for initiating gene expression.

Serratia spp. have been found to produce a number of hydrolytic enzymes which are excreted into the culture medium. This is in contrast to other gram-negative bacteria, in which proteins are preferentially excreted to the periplasmic space rather than to the surrounding medium. Such periplasmic proteins tend to leak into the culture medium, especially when the cells are grown to high densities.

According to the present invention, DNA encoding extracellular Serratia spp. enzymes (that is, extracellular when expressed in Serratia), has been isolated, and microorganisms suited for the industrial production of gene products and harbouring the Serratia DNA have been grown and have been found to produce the Serratia enzymes.

It was also found that when hybrid plasmids containing inserted DNA encoding an extracellular Serratia spp. enzyme were harboured by another microorganism which, in itself, does not usually excrete its gene products into the culture medium, i.e. *E. coli*, the Serratia enzyme was, to some extent, excreted by *E. coli* into the culture medium (cf. Examples 1 and 6). It is therefore possible to partially purify the portion of the Serratia enzyme excreted into the culture medium from the *E. coli* cells in a relatively simple way, for instance by filtration to remove the *E. coli* cells, and precipitation of the enzymes from the filtrate, for instance with ammonium sulphate. In the present context, the term "excrete" is understood to mean transport of a gene product through at least the cytoplasmic membrane of the cell.

Thus, one aspect of the invention relates to a method of producing a bacterial enzyme, comprising cultivating, in a culture medium, a microorganism harbouring a hybrid plasmid which contains DNA from Serratia spp. encoding an extracellular Serratia spp. enzyme, and harvesting the enzyme from the culture.

In a particular embodiment, the invention relates to a method for producing Serratia spp. enzymes substantially free from other bacterial proteins, in which a portion of the enzyme is excreted from the microorganism into the culture medium and harvested from the culture medium.

The cultivation of the microorganism is preferably performed in a liquid culture medium containing the nutrients and minerals required for the optimal growth of the microorganism. The harvesting of the enzyme may be performed in a manner known per se. As mentioned above, the purification of the enzyme may be performed by filtration to remove the host cells, and precipitation of the nuclease from the filtrate. Normally, the precipitate is then dissolved in a suitable buffer, e.g. Tris-EDTA, followed by dialysis to remove the precipitation agent.

Examples of hydrolytic enzymes produced by Serratia spp. are a nuclease which hydrolyzes nucleic acids into nucleotides, oligonucleotides, or smaller nucleic acid fragments, and a lipase and a phospholipase which hydrolyze fatty acids from lipids and phospholipids.

The microorganism is typically a bacterium, preferably a gram-negative bacterium. It is normally not desirable to employ Serratia spp. for the production of the Serratia spp. enzymes, as Serratia spp. are opportunistic pathogenic bacteria, which may limit their utility as production microorganisms. Furthermore, Serratia spp. produce an extracellular protease which may contaminate the desired product. The preferred gram-negative bacteria for use as production microorganisms for the production of the Serratia spp. enzymes are bacteria generally employed for the production of gene products such as *E. coli*.

The invention also relates to a hybrid plasmid which carries DNA from Serratia spp., encoding an extracellular Serratia spp. enzyme as described above.

Plasmids useful as vectors for the production of the enzymes according to the invention may be any type of plasmid usually employed for this purpose which is able to replicate in the microorganism in question. Plasmids which may be used to produce large quantities of the enzymes in question are, e.g., the so-called runaway plasmids, that is, plasmids with a conditionally uncontrolled replication behaviour. Plasmids showing this behaviour are disclosed, in, for instance, U.S. Pat. No. 4,495,287 and European Patent Application, Publication No. 0109150.

Bacterial nucleases are enzymes which are of considerable value in the purification of, e.g., proteinaceous products prepared by the fermentation of microorganisms such as products prepared by the fermentation of cells modified by recombinant DNA techniques and producing products not naturally associated with the cell in question. An important step in the purification of these products is to separate the proteinaceous products from nucleic acid derived from the cells. This purification, when performed by standard chemical treatments such as precipitation of the nucleic acids, incurs a risk of loss of the desired product produced by the cells due to the high viscosity of the material containing the desired product which renders separation thereof difficult, whereas the decomposition of the nucleic acids by means of nuclease does not incur any substantial loss of the desired product. Also, the efficient and complete removal of nucleic acids from the products is important, e.g., when the products are to be used for administration to human beings, as it is a requirement by health authorities in some countries that the product should not contain any hybridizable DNA from the cells employed to produce the product in question.

Therefore, a highly interesting enzyme produced by Serratia spp. is a nuclease which has been found to be very potent and which is of great industrial importance for the removal of nucleic acids from a biological material. In the present context, the term "removal of nucleic acids" is intended to indicate that long nucleic acid sequences are degraded to shorter fragments or oligonucleotides or, in some cases, to mono- or dinucleotides. This means that the products resulting from the nuclease action are rather easy to remove by conventional separation methods.

Accordingly, the present invention further relates to a bacterial nuclease which is a Serratia spp. nuclease with the following amino acid sequence (deduced from the DNA sequence in a manner known per se, and including the N-terminal signal peptide):

MetArgPheAsnAsnLysMetLeuAlaLeuValAlaLeuLeuPheAlaAlaGlnAlaSerAlaAsp
ThrLeuGluSerILeAspAsnCysAlaValGlycysProThrGlyGlySerSerAsnValSerIleValArg
HisAlaTyrThrLeuAsnAsnAsnSerThrThrLysPheAlaAsnTrpValAlaTyrHisIleThrLysAsp
ThrProAlaSerGlyLysThrArgAsnTrpLysThrAspProAlaLeuAsnProAlaAspThrLeuAlaProAla
AspTyrThrGlyAlaAsnAlaAlaLeuLysValAspArgGlyHisGlnAlaProLeuAlaSerLeuAlaGly
ValSerAspTrpGluSerLeuAsnTyrLeuSerAsnIleThrProGlnLysSerAspLeuAsnGlnGlyAla
TrpAlaArgLeuGluAspGlnGluArgLysLeuIleAspArgAlaAspIleSerSerValTyrThrValThr
GlyProLeuTyrGluArgAspMetGlyLysLeuProGlyThrGlnLysAlaHisThrIleProSerAlaTyr
TrpLysValIlePheIleAsnAsnSerProAlaValAsnHisTyrAlaAlaPheLeuPheAspGlnAsnThr
ProLysGlyAlaAspPheCysGlnPheArgValThrValAspGluIleGluLysArgThrGlyLeuIleIle

TrpAlaGlyLeuProAspAspValGlnAlaSerLeuLysSerLysProAlaSerCysArgSer |.

The enzyme may, for instance, be produced by the method described above.

For special applications, such as when a nuclease is to be used for removing residual nucleic acids from an otherwise substantially purified biosynthetic product (as described in further detail below), the enzyme should preferably be in substantially pure form. In order to obtain the substantially pure enzyme, a crude enzyme preparation may be partially purified by ultrafiltration or precipitation with, e.g. ammonium sulphate, and subjected to further purification by, for instance, chromatography (such as ion exchange chromatography or affinity chromatography) or preparative gel electrophoresis. In some cases, it will be an advantage to provide the enzyme in immobilized form on a suitable matrix as this may facilitate an easy removal of the nuclease after use and also makes it possible to use the enzyme once more. Examples of such matrix materials are dextran or agarose gels or an inorganic material such as a siliceous material, e.g., silica and silicic acid and derivatives thereof. The immobilization may be performed in a manner known per se.

Another enzyme of potential interest is a phospholipase produced by Serratia spp. The present invention therefore relates to a Serratia spp. phospholipase encoded by the following DNA sequence:

ATGAGTATGCCTTTAAGTTTTACCTCTGCAGTATCCCCGGTGGCCGCGATCCCTACGCCTCGCGCTGCCGAGACGCGGACGG
TACTCATACGGAAATTCAAAATGGAGACGTCATAGGGGCCACCGGCGCTAGGGATGCCGGAGCGCGCCGACGGCTCTGCGCCTGCC

CGGCGAGCCTGCGGCACGCCGGCAAATCCGGGCCGGTGGCCTCTCCCTCTCAGAACACGCTGAACGCGCAGAATCTGTTGAATAC
GCCGCTCGGACGCCGTGCGGCCGTTTAGGCCCGGCCACCGGAGAGGGAGAGTCTTGTGCGACTTGCGCGTCTTAGACAACTTATG

GCTGGTCGGCGATATCTCAGCGGCGGCACCGACGCGCCGGGCGTGACGCGGGGGCAGCAATCGCAGGAGAGGGGGATT
CGACCAGCCGCTATAGAGTCGCCGCCGTGGCTGCGCGGCCCGCACTGCGCCCCCGTCGTTAGCGTCCTCCCCCCTA

TATGCGTTGGCGCTGTTGGCCAAGGACGTTTACTCACTCAATGGCCAGGGCGCCGCCGGGTTCAACCGCCTGAGCGACACCGCTG
ATACGCAACCGCGACAACCGGTTCCTGCAAATGAGTGAGTTACCGGTCCCGCGGCGGCCCAAGTTGGCGGACTCGCTGTGGCGAC

CTCGGTTTCGGCATCGATCCCGCCAGCCTGCACGACGCGGGCAGCGGTTTCCCAGGCTGGGATTTACAGCAACGACAAACAGTAT
GAGCCAAAGCCGTAGCTAGGGCGGTCGGACGTGCTGCGCCCGTCGCCCAAAGGGTCCGACCCTAAATGTCGTTGCTGTTTGTCATA

GTGTTGGCGTTCGCCGGCACCAACGACTGGCCGCGATTGGCTGAGCAACGTGCGGCAGGCGACGGGCTATGACGATGTGCAGTAC
CACAACCGCAAGCGGCCGTGGTTGCTGACCGGCGCTAACCGACTCGTTGCACGCCGTCCGCTGCCCGATACTGCTACACGTCATG

AATCAGGCGGTTGCCGCTGCCAAAAGCCGCCAAGGCGGCCTTCGGCGATGCGCTGGTGATCGCCGGCCATTCGCTTGGCGGTGGT
TTAGTCCGCCAACGGCGACGGTTTTCGGCGGTTCCGCCGAAGCCGCTACGCGACCACTAGCGGCCGGTAAGCGAACCGCCACCA

CTGGCGGCCACCGCCGCGCTGGCGACCGGCACCGTCGCGGTCACCTTCAACGCGGCCGGGGTCTCGGATTACACCCTGAATCGCCT
GACCGCCGGTGGCGGCGCGACCGCTGGCCGTGGCAGCGCCAGAGCCTAATGTGGGACTTAGCGGA

GGGCATCGATCCGGCGGCAGCGAAGAAAGATGCCGAAGCCGGCGGCATTCGCCGTACAGCGAGCAATATGACATGCTGACCAGCA
CCCGTAGCTAGGCCCGCCGTCGCTTCTTTCTACGGCTTCGGCCGCCGTAAGCGGCATGTCGCTCGTTATACTGTACGACTGGTCGT

CCCAGGAGTCGACCTCGCTGATCCCGGATGCCATCGGCCACAACATCACCCTGGCCAACAACGATACCCTGACCGGCATCGATGA
GGGTCCTCAGCTGGAGCGACTAGGGCCTACGGTAGCCGGTGTTGTTGCTATGGGACTGGCCGTAGCTACT

CTGGCGGCCGAGCAAACATCTGGATCGCAGCCTGACGGCGCACGGCATCGACAAGGTGATAAGCTCGATGGCGGGAACAAAAGCCG
GACCGCCGGCTCGTTTGTAGACCTAGCGTCGGACTGCCGCGTGCCGTAGCTGTTCCACTATTCGAGCTACCGCCTTGTTTTCGGC

TGGGAGGCGAAGGCCAATGCCTGA
ACCCTCCGCTTCCGGTTACGGACT

In a further aspect, the invention relates to a composition for removing nucleic acids from a biological material, which composition comprises a Serratia spp. nuclease. In the present context, the term "biological material" is understood to indicate any material in which at least one component is of biological origin. The term is therefore intended to include a solution of nucleic acids alone (for instance originating from in vitro laboratory work), a fermentation medium containing a cell culture producing a biosynthetic product, a fermentation medium in which a cell culture producing a biosynthetic product has been grown (and which may therefore contain this product as well as nucleic acids originating from spontaneous cell rupture), or a resuspension of a cell culture, which produces a biosynthetic product, after the cells have been harvested from the medium, e.g. by centrifugation, the cell culture comprising either whole or lysed cells.

The term "biosynthetic product" is understood to mean a product which may be a protein, polypeptide, glycolipid carbohydrate or low molecular weight compound. Nucleic acids are particularly important contaminants when the biosynthetic product is not excreted from the cell, necessitating cell lysis in order to harvest the product, in that they impart viscosity to the cell lysate to such an extent that the purification of the product is rendered difficult. To reduce the viscosity of a cell lysate, it is therefore advantageous to provide a composition which contains a nuclease such as a Serratia spp. nuclease of the invention. The nuclease may, for instance, have the amino acid sequence shown above. The nuclease composition of the invention should preferably be substantially free from proteolytic activity as the presence of proteases in a composition of this nature would be a most serious cause of degradation of the proteinaceous products produced by the cell culture. The nuclease prepared by the method of the present invention, the gene coding for which has been obtained from a Serratia spp. organism has in fact been found to be substantially free from proteolytic activity (see Example 2); it should be mentioned that a substantially protease-free composition is particularly important when the composition is to be used to remove residual nucleic acids from an otherwise purified proteinaceous product since, when the nuclease is added to an unpurified cell lysate, the proteolytic activity of the lysate itself will far exceed any proteolytic activity remaining in the nuclease composition. The substantially protease-free nuclease composition is therefore particularly advantageous to use (in substantially pure form, of course) in connection with proteinaceous products which have already undergone several purification steps.

Experiments have shown that even when an excess of nuclease is added to a cell lysate (excessive to a reduction of the viscosity ascribable to the nucleic acid components of the lysate), a minor fraction of nucleic acids may remain to contaminate the proteinaceous product. This is believed to be the result of a "masking" of the nucleic acids, for instance through interactions of nucleic acids with membrane and/or protein components of the lysate. However, complete removal of nucleic acids (defined as the absence of nucleic acids hybridizable by DNA or RNA probes) is often required by the health authorities in several countries (e.g. the FDA) when the biosynthetic products produced by recombinant DNA techniques or from tissue cultures are to be used for medical purposes. When such products are to be used for other purposes where the presence of even minute amounts of nucleic acids might interfere with the desired result, the complete removal of residual nucleic acids is also highly desirable. The present inventors have found that such residual nucleic acids may be completely removed when certain detergents or protein denaturing agents are added together with the nuclease. For applications requiring the complete removal of nucleic acids, it is therefore advantageous that the composition of the invention comprises a nuclease, such as a Serratia spp. nuclease, together with a detergent and/or a chaotropic agent. The detergent may, for instance, be a non-ionic detergent such as a polyoxyethylene alcohol, e.g. Brij ® 58 or an octoxynol, e.g. Triton ® X-100, or an ionic detergent such as sodium dodecyl sulphate (SDS) or a deoxycholate such as sodium deoxycholate. The chaotropic agent may be selected from urea, thiourea or a salt of thiocyanic acid.

In a still further aspect, the invention relates to a method of removing nucleic acids from a biological material (as defined above), in which a Serratia spp. nuclease is added to the biological material. More particularly, the method of the invention is useful in a variety of situations where contamination with nucleic acids is a problem, such as where the biological material comprises a waste solution or suspension of nucleic acids resulting, for instance, from in vitro experiments with nucleic acids and contaminating laboratory equipment; where the biological material comprises a fermentation medium containing a cell culture producing a biosynthetic product (as defined above), in which case the nuclease may be added before or after cell lysis in a sufficient quantity to secure the removal of the bulk of the nucleic acids in the material; where the biological material comprises a fermentation medium in which a cell culture producing a biosynthetic product has been grown and from which the cells have subsequently been removed, in which case the medium may contain a certain amount of nucleic acids due to spontaneous cell rupture and optionally a biosynthetic product excreted from the cells into the medium; and where the biological material comprises a resuspension of a cell culture producing a biosynthetic product after the removal of the fermentation medium, in which case the nuclease may be added before or after cell lysis. The nuclease may be the one which has the amino acid sequence shown above. The present inventors have found that particularly advantageous results may be obtained when the nuclease of the invention is added to the biological material prior to cell lysis. Experiments have demonstrated that a high degree of reproducibility with respect to the elimination of the viscosity of lysates of, for instance, *E. coli* (such as freeze-thaw lysates and French Press lysates) is obtained when the nuclease is added to the cell culture (suspended or in medium) prior to cell lysis. Also, a shorter period of time (on the order of minutes rather than hours) and a lower temperature level have unexpectedly been found to be required to attain a certain relative viscosity than when the nuclease is added after cell lysis, which results in a higher yield of the biosynthetic product (for instance, less degradation of a proteinaceous product during removal of nucleic acids).

Many health authorities require that recombinant organisms must be killed prior to being released from the closed fermentation system. In many cases, this is accomplished by adding phenol and toluene during the last phase of the fermentation. It has been found that the nuclease of the present invention retains its activity in

the presence of the quantities of phenol and toluene required to kill the cells in the fermentor.

When adding the nuclease to a biological material in accordance with the invention in order to reduce the viscosity of the material, the end product of the nuclease action includes differently sized nucleic acid fragments and oligonucleotides rather than mono- or dinucleotides only. For certain purposes, for instance when it is desired to produce a highly purified end product from which all hybridizable nucleic acids have been removed, it is recommended to add the enzyme to a product which has already been purified, i.e. at least substantially separated from other components of the biological material.

As mentioned above, it has been found that residual nucleic acids, i.e. nucleic acids which remain in a biological material after a limit digest (where nuclease has been added in such excess in order to reduce the viscosity of the material that no further addition of nuclease will reduce the amount of nucleic acids still further), constitute a minute fraction, in fact less than 0.1% of the total amount of nucleic acids in a given biological material and represent nucleic acids which are ordinarily inaccessible to the nuclease due to interactions with membrane components and/or proteins as discussed above. It has been found that if the nuclease treatment is carried out in the presence of a detergent and/or a chaotropic agent, the residual nucleic acids can be digested.

Thus, the invention further relates to a method of removing residual nucleic acids from a biosynthetic product, in which the nuclease is added in the presence of a detergent and/or a chaotropic agent in order to digest the nucleic acids present as oligonucleotides or nucleotides which cannot be detected by hybridization. The detergents and chaotropic agents most likely act by counteracting the hydrophobic and electrostatic forces which are responsible for the formation of a complex structure in which segments of nucleic acids remain inaccessible to the nuclease.

The detergents and chaotropic agents selected should be ones which do not permanently damage the secondary and tertiary protein structure of any desired proteinaceous product present in the biological material, i.e. a substance which may be removed after the nuclease has acted in its presence to remove residual nucleic acids in such a way that the correct structure of the product is obtained. Such detergents and chaotropic agents may, for instance, be the ones mentioned above. When employing a detergent or chaotropic agent, care should also be taken not to incorporate such substances in such amounts that the nuclease activity will be impaired or even eliminated. When the detergent is a non-ionic detergent, it is usually added in an amount of 0.2–1.5%, in particular about 0.4–1.0%, of the biological material. When it is an ionic detergent, it is generally added in an amount of 0.01–1.0% of the biological material. The chaotropic agent is usually added in an amount of 2–8M (about 10–50% w/v of the biological material).

In order to obtain a product which is completely free from nucleic acids, it may be an advantage to first employ the nuclease of the invention at an early stage of the production process in order to reduce the viscosity from a cell lysate and remove the bulk of the nucleic acids present in it. In a subsequent step in the purification procedure, the purified nuclease may be employed in solution or in immobilized form in order to remove any residual nucleic acids from the product.

It is further contemplated that a nuclease-containing composition of the invention may be used to remove the infectious potential of infectious agents either as a means to ensure the elimination of the infectious potential itself or as a means to recover such components of these agents as might be desired to produce vaccines or diagnostic agents. In the present context, the term "infectious agent" is understood to mean a living or non-living agent the infectious potential of which is ascribable to nucleic acid components. These nucleic acid components may encode RNA species and/or proteins essential for the infectious potential (they may, for instance, be needed for propagation), or they may play a purely structural role in the infectious agent. Infectious agents may accordingly include plasmids, viruses, bacteria, prions and parasites.

The infectious potential of these agents may, in some cases, be destroyed by means of chemicals, but it may be an advantage in many cases to use a nuclease for decontamination purposes. Free DNA molecules such as plasmids liberated from cells during growth, may for instance readily be digested by means of the nuclease of the invention, which is also the case with potentially infectious DNA present in waste material from laboratory experiments. As a safety precaution, it may often be desired also to remove nucleic acids from the waste material resulting from the industrial production of biosynthetic products by recombinant DNA techniques. If the nucleic acid component of the infectious agents present in such waste is not freely accessible to the nuclease of the invention, the simultaneous addition of a detergent or chaotropic agent may be recommended as described above in order to remove all nucleic acids present.

A further contemplated use for the nuclease of the invention is in the production of antigens and vaccines. At present, attenuated strains of bacteria and viruses are usually employed to elicit an immunological response to more virulent members of the same species, one important advantage being the preservation of the integrity of complex antigenic structures on the surface of or inside the infectious agent during the limited period of propagation of the agent in vivo. By using the nulease of the invention, it would be possible to preserve the antigenic complexity, permitting the immunological response to be directed against any strong antigenic determinants associated with the infectious agent in question, while avoiding the risk of vaccination sequelae occasionally seen with live vaccines using attenuated organisms. The nuclease of the invention could be used to remove nucleic acid components of such infectious agents, optionally together with a detergent and/or a chaotropic agent to make the nucleic acids available to the nuclease, the detergent or chaotropic agent and the concentration in which they are used being so selected that it does not interfere with the antigenic structure in question.

The Serratia spp. hydrolytic enzymes, produced by the method of the invention, have been found to be expressed at a late stage in the growth cycle of microorganisms producing the enzymes, whether these were Serratia spp. or *E. coli*. As shown in the Examples, this late expression is a result of the gene expression regulating behaviour of a regulatory region from which expression or the genes in question is initiated. Thus, during most of the exponential growth of the culture, little or no hydrolytic enzyme is synthesized, whereas a high rate of gene expression occurs when the cells enter the

late exponential growth phase. In the present context, the term "regulatory region" is understood to mean a molecular sequence involved in the transcriptional control of a gene comprising such sequences as the promoter, any binding sites for regulatory proteins (regulating gene expression), e.g. cyclic AMP binding protein (CAP), and sequences of yet unknown function in transcriptional control, but found, by deletion mapping, to be of importance for transcriptional control.

This regulatory principle may be utilized in accordance with the present invention to provide a plasmid comprising a regulatory region from which expression of a gene located downstream of the regulatory region is initiated or increased at a late stage in the growth cycle of the microorganism harbouring the plasmid. The gene may be one which is not naturally related to the regulatory region.

A regulatory mechanism as described above where gene expression is initiated or increased at a late stage in the growth cycle of the microorganism, is often advantageous and desirable for production cultures. Thus, in a fermentation process, the high cell density occurring late in the fermentation is the potentially most productive period of the culture, and during this period, it may be of great value to have a high rate of gene expression. This is normally not obtained using the known promoters because their activity usually follows the growth rate of the culture, and is therefore minimal at the stage where cell density is highest. The special behaviour of the regulatory regions found in Serratia spp. genes may also be of particular value in cases where the products to be produced by the culture are toxic to the microorganism in question, as the microorganism will only synthesize the toxic product when growth has already or nearly stopped.

Consequently, the present invention further relates to a plasmid which comprises a regulatory region from which expression of a gene located downstream of said regulatory region is initiated or increased at a late stage in the growth cycle of microorganisms harbouring the plasmid. Such a regulatory region is particularly useful for regulating the expression of a gene not naturally related to the regulatory region, such as when the plasmid carrying the regulatory region is to be employed as a cloning or production vector with the object of obtaining, by fermentation of a microorganism harbouring the plasmid, a wide variety of biosynthetic products for technical or medical purposes. Examples of such biosynthetic products are polypeptides and proteins or fragments thereof, enzymes and non-proteinaceous products of reactions of enzymes with a compound in the nutrient medium, low molecular weight products such as hormones, and nucleic acids; products which are contemplated to be of particular interest are products of eucaryotic, especially mammalian genes and, as mentioned above, products which are toxic to the microorganism in which they are produced.

The regulatory region may be one which is found in Serratia spp. genes, although it is contemplated that similar regulatory regions may also be found in other organisms. In particular, the regulatory regions is a nuclease or phospholipase regulatory region from Serratia spp. examples of which are shown in FIG. 7, position **1–385**, and FIG. 9, position **201–415**, respectively.

A regulatory region as described above may be inserted into any known or new cloning or production vector by means of standard recombinant DNA techniques.

Particularly interesting plasmids useful as cloning or production vectors containing the above-mentioned type of regulatory region are the so-called runaway plasmids, that is, plasmids with a conditionally uncontrolled replication behaviour. Plasmids showing this behaviour are disclosed in, for instance, U.S. Pat. No. 4,495,287 and European Patent Application, Publication No. 0109150.

The strength of the promoter included in the regulatory region of, e.g., the nuclease gene may not always be sufficient for certain production purposes, and therefore the ability of the regulatory region to give rise to growth phase related expression of a gene located downstream from the regulatory region may be further exploited by replacing the present promoter with a stronger constitutive promoter in such a way that the growth phase dependent expression is preserved.

Apart from employing the regulatory region for the expression of a biosynthetic product, a particularly interesting application of the regulatory region is to utilize it to increase transcription of a gene located downstream of the regulatory region, which gene is involved in the control of replication of a bacterial plasmid thereby causing uncontrolled plasmid replication (so-called runaway replication) at a late stage in the growth of cells harbouring the plasmid. Most runaway replication vectors described so far (cf. for instance European Patent Application, Publication No. 0109150) require external manipulation of the growth conditions, e.g., an increase in temperature, to initiate uncontrolled replication. By using the regulatory regions described above to regulate plasmid replication, a novel approach has become possible, namely the initiation of runaway replication as a function of the growth phase of cells harbouring the plasmid. This approach is advantageous from three points of view. Firstly, no external manipulation of the growth conditions is required, secondly, no specific properties of the host cells are required to initiate runaway replication, and thirdly, uncontrolled replication is initiated at a time when the microbial culture enters the late exponential growth phase, that is, when the effect of increasing the copy number of a gene to be expressed is greatest. A preferred regulatory region for initiating runaway replication in the late exponential growth phase is the phospholipase regulatory region due to its dual control systems. One regulatory system ensures that expression of a gene controlled by the phospholipase regulatory region is restricted to the late exponential growth phase; the other regulatory system is able to override the first control system and comprises a glucose repression system.

In the practical exploitation of the regulatory region described above, a DNA fragment carrying both regulatory systems from the phospholipase regulatory region may be inserted into a plasmid upstream of a replication regulatory gene or genes, the plasmid may be transformed to a suitable host microorganism, and transformants may be selected in the presence of glucose. When these transformants are deprived of glucose, they will exhibit the runaway replication phenotype during the late exponential growth phase. A gene expressing a desired biosynthetic product may subsequently be inserted into the plasmids thus produced, the resulting hybrid plasmids may be transformed to a suitable host microorganism, and the host may be grown to a production size culture either in the absence of glucose or in the presence of glucose in such an amount that it is consumed by the cells before they enter the late

exponential growth phase; in either case, uncontrolled replication is initiated in the late exponential growth phase due to increased transcription from the regulatory region. The biosynthetic product is harvested from the culture after a suitable period of time to ensure a sufficient production of the product. Apart from the specifics given above, the cultivation is suitably performed using conventional techniques, including conventional nutrient media which are known to be optimal to the microbial species used as the host. Also, the harvesting of the biosynthetic product is performed in accordance with well-known methods adopted to the identity and properties of the particular biosynthetic product, the properties of the host, etc.

The present invention also provides a microorganism harbouring a plasmid which carries a regulatory region as specified above. The microorganism is typically a bacterium such as a gram-negative bacterium, and preferred gram-negative bacteria are ones which are generally employed for the production of biosynthetic products, for instance *E. coli.*

It is further contemplated that the sequence encoding the N-terminal part of the nuclease, which sequence is indicated to encode a signal peptide essential for transmembrane transport of the nuclease, may be employed to obtain excretion of a gene product. A sequence coding for a desired biosynthetic product may be combined directly with the sequence specifying the C-terminus of the signal peptide of the nuclease thus allowing the desired protein to be excreted, the signal peptide being removed in the process. For practical purposes, the sequence coding for the signal peptide (cf. FIG. 7) may be isolated together with the nuclease regulatory region as a DNA fragment extending from position **1** to **448**, with latter position conveniently corresponds to the recognition site for AhaIII and which precisely corresponds to the last codon of the signal peptide (including the signal peptidase recognition site). The DNA fragment may subsequently be inserted into any suitable vector and ligated at the "filled in" (by means of Klenow polymerase) AhaIII site to a sequence coding for a product to be excreted. The optional presence of the nuclease regulatory region further allows the expression to be limited to the late stages of cell growth.

DESCRIPTION OF THE DRAWINGS

The invention is further explained below with reference to the drawings in which

FIG. **1** shows a linear restriction enzyme and genetic map of the hybrid plasmid pNU121-nuc+ carrying the nuclease gene (Nuc) of *Serratia marcescens* W225. The symbols used are: structural genes ■ →=promoters. Ap=ampicillin resistance; Tc=tetracycline resistance; C_I=lambda repressor gene, λpR=lambda promoter. P=PstI; E1=EcoRI; E5=EcoRV; F2=FnuDII.

FIG. **2** shows the time course of nuclease treatment of X-PRESS lysate of *E. coli.* Ordinate: relative viscosity (H_2O at 0° C. as reference). Abscissa: hours of incubation at 0° C. following X-PRESS lysis.

FIG. **3** shows the time course of nuclease treatment of French Press lysate of *E. coli.* Ordinate: relative viscosity (H_2O at 0° C. as reference). Abscissa: hours of incubation at 0° C. following French Press lysis.

FIG. **4** shows the time course of nuclease treatment of French Press lysate of *E. coli.* Left column indicates the concentration of nuclease (U/ml) added before or after cell lysis. Six samples were followed individually in time course experiments (minutes of incubation at 0° C.); zero time corresponds to release from French Press. Visual estimation of viscosity was carried out from 0–70 minutes as indicated in each line, cfr. symbols given below lines. The relative viscosity (H_2O at 0° C. as reference) was measured at 70 minutes and 15 hours of incubation at 0° C.

FIG. **5** shows the relationship between relative viscosity (H_2O at 0° C. as reference) (ordinate), concentration of nuclease (abscissa), and duration of incubation at 0° C. The figures is a representation of the data given in FIG. **5**. Note that the abscissa is log-scale.

FIG. **6** shows the agarose gel electrophoretic pattern of non-digested nucleic acid present in the digest when the viscosity was usually estimated as "aqueous". Samples were taken from the digests shown in FIG. **5**.

FIG. **7** (comprising *7a* and *7b*) shows the nucleotide sequence of the 1.3 kB DNA fragment (F2-fragment shown in FIG. **1**) carrying the nuclease gene from Serratia W225.

FIG. **8** shows a linear restriction enzyme and genetic map of the hybrid plasmid pNU121-phl+ consisting of the 4.5 Kb vector pNU121 and an insert of 3.2 Kb Serratia spp. A1 DNA containing the gene of the phospholipase operon. → indicates the promoter of the gene and the direction of transcription, ■ indicates a structural gene, Ap and Tc denote the genes for ampicillin and tetracycline resistance, respectively, C_I denotes the λ repressor gene. Restriction enzymes: E_1=EcoRI, E_5=EcoRV, P=PstI, Sa=SalI, Sm=SmaI, N=NarI, H_3=HindIII, Bc=BcII, Ba=BamHI.

FIG. **9** (comprising *9a–9d*) shows the DNA nucleotide sequence of 1.6 Kb of the 3.2 Kb Serratia spp. A1 DNA containing the phospholipase (phl) gene. The positions of a few restriction sites are indicated, CAP with underlined sequences indicates the position of the putative catabolite activator binding site and regulatory region of the phospholipase gene, S.D. indicates the position of a Shine-Dalgarno homology for the ribosomal binding site. The gene starts at position **416** and ends at position **1372**.

MATERIALS AND METHODS

The strains of *Escherichia coli* K-12 and *Serratia marcescens* W225 are listed in Table 1. Plasmids and bacteriophages used are listed in Table 2.

TABLE 1

	Bacterial Strains Used	
Bacterial Strains	Genotype	Reference/Source
E. coli K-12 MT102	thi, ara-levΔ7679, ara D139, lacΔ × 74, galU, galK, rpsL, hsdR.	
E. coli K-12 CSH50	Δpro-lac, rpsL	J. Miller: Experiments in Molecular genetics, CSH Lab., Cold Spring Harbor, 1972.
E. coli K-12 W3110	tna trp	S. G. Shogman & J. E. Sjöström, J. Gen. Microbiol. 130, 1984, p. 3091.
E. coli K-12 JM103	Δlac pro, thi, strA supE, endA, sbcB15, hsdR4, F'tra D36, proAB, lacI^q zΔM15	J. Messing, Nucl. Acids Res. 9, 1981, pp. 309–321
E. coli K-12 S17	thi, pro, hsdR, hsdM+, recA	R. Simon, Bio/Technology, November 1983
Serratia marcescens W255	Tc^R	U. Winkler, Molec. gen. Genet. 124, 1973,

TABLE 1-continued

Bacterial Strains	Bacterial Strains Used: Genotype	Reference/Source
		pp. 197–206.

TABLE 2

Name	Plasmids and Bacteriophages Used: Relevant Phenotype	Reference/Source
pNU121	Ap^R, pBR322 derivative	B. Nielsson: Nucl. Acids Res. II, 1983, pp. 8019–8030
pOU57	RI "runaway replication" derivative, Ap^R	J. E. L. Larsen, Gene 28, 1984, pp. 45–54
pGV403	Cm^R, pBR322 derivative	Amersham Ltd.
pACYC177/ cI857	Kan^R, derivative of pACYC177 carrying λcI857	Chang & Cohen, J. Bacteriol. 134, 1978, pp. 1141–1156
pLc28	Ap^R, pBR322 derivative	E. Remaut et al., Gene 15, 1981, p. 81
M13 mp8 and mp9	Phage M13 derivatives for DNA nucleotide sequencing	Amersham and J. Messing. Nucl. Acids Res. 9, 1981, pp. 309–321

All experimental techniques used were standard techniques as described in T. Maniatis: *Molecular Cloning*, Cold Spring Harbor Laboratory, 1982, and J. Miller: *Experiments in Molecular Genetics*, Cold Spring Harbor, 1972.

All cells were grown in LB medium (Bertani, *J. Bact.* 62, 1951, p. 293) or in A+B minimal medium (Clark and Maaløe, *J. Mol. Biol.* 23, 1967, p. 99), with addition of vitamins and amino acids. Plates for bacterial growth contained LB medium and 1.5% agar with or without antibiotics: Tetracyclin 8 μg/ml, ampicillin 50 μg/ml, chloramphenicol 20 μg/ml. Plates for screening for nuclease activity contained DNase test agar (Difco) for DNase activity.

EXAMPLE 1

Preparation of chromosomal DNA from *Serratia marcescens* W225

A culture of *Serratia marcescens* W225 was deposited in the DSM (Deutsche Sammlung von Mikroorganismen, Grisebachstrasse 8, D-3400 Göttingen, West Germany) on May 8, 1985 under the Accession No. 3308). The culture was grown overnight in LB medium and harvested by centrifugation (8,000 r.p.m. for 5 minutes). The cells were washed twice in TEN-buffer (10 mM Tris, HCl, pH 8, 1 mM EDTA, 100 mM NaCl) and resuspended in 20 ml TEN-buffer containing 1 mg/ml lysozyme and 0.1 mg/ml RNase. The cells were incubated at 37° C. for a period of 30 minutes and 20% SDS was added to a final concentration of 1%. After 60 minutes at a temperature of 37° C. (for total lysis), the lysate was incubated at a temperature of 4° C. overnight. Next day the cell debris was removed by centrifugation (18,000 r.p.m. for a period of 25 minutes). The supernatant was transferred to a new tube containing 2 ml 3M sodium acetate and 2 volumes of isopropanol. Upon gentle mixing, the DNA precipitated in threads which were picked up by means of a curved glass needle. The precipitated DNA was washed twice in 80% ethanol and resuspended in TEN-buffer. The DNA was further purified by buoyant density gradient centrifugation, and after appropiate dilution it was extracted with phenol and dialysed against TE-buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). Finally, the DNA was tested for absence of nuclease by incubation at 37° C. with restriction enzyme buffer.

Construction of a gene bank from *Serratia marcescens* W225.

The cloning vector plasmid, pNU121, was used in connection with the construction of a gene bank from *Serratia marcescens* W225. The plasmid is a pBR322 derivative coding for both ampicillin resistance and tetracyclin resistance, but the promoter of the tetracyclin resistance gene is replaced by the phage λ promoter, λpR, and since the λ repressor gene, C_I, is also present on pNU121, tetracyclin resistance is normally not expressed. Resistance is, however, expressed if the C_I gene is destroyed by insertion of DNA into the C_I gene.

Therefore, pNU121 DNA having a unique EcoRI site in the C_I gene was digested with the restriction enzyme EcoRI and mixed with *Serratia marcescens* DNA partially digested with EcoRI. The DNA was ligated at 15° C. overnight with T4 ligase and transformed to *E. coli* strain MT102. Selection was made at 37° C. on LB plates containing 8 μg/ml tetracyclin, so only cells harbouring pNU121 with inserted DNA will give rise to colonies. Approximately 2,500 colonies representing a gene bank of *Serratia marcescens* W225 were isolated by this procedure.

Isolation of a nuclease gene from *Serratia marcescens* W225

The gene bank from *Serratia marcescens* W225 was replica plated onto DNase indicator plates (see Materials and Methods) and after growth at 37° C. for two days, the plates were developed with 0.1N HCl. DNase positive colonies were surrounded by a clearing zone. One positive clone, pNU121-nuc+, was re-isolated from the master plate and tested for the presence of other genes coding for extracellular enzymes. (*Escherichia coli* MT102/pNU121-nuc+ was deposited in the DSM on May 8, 1985 under the Accession No. 3309.) The clone was found to express RNase too, but no other extracellular enzymes were expressed from the clone. The EcoRI fragment carrying the nuclease gene was also inserted into the runaway cloning vector pBEU50 resulting in the plasmid pBEU50-nuc+. (*Escherichia coli* C600/pBEU50-nuc+ was deposited in the DSM on May 8, 1985 under the Accession No. 3310.)

Restriction enzyme mapping of the nuclease gene

Plasmid DNA from *E. coli* strain MT102 harbouring the nuclease gene was prepared and digested with the restriction enzymes EcoRI, PstI and EcoRV, respectively. The digested fragments were analyzed by agarose gel electrophoresis resulting in the map shown in FIG. 1. The DNA digested with PstI was religated with T4 DNA ligase and transformed to strain MT102. Selection was made on DNase indicator plates containing 8 μg/ml tetracyclin. After incubation, the plates were developed and all colonies showed a nuclease positive phenotype. When the DNA digested with EcoRV was religated and transformed to MT102, selecting for ampicillin resistance, all transformants were nuclease negative. Therefore, the nuclease gene is carried on a 2 Kb PstI-EcoRI fragment as shown in FIG. 1.

For further subcloning, the plasmid DNA was digested with both PstI and EcoRI, and after electrophoresis the PstI-EcoRI fragment carrying the nuclease gene was purified from the gel. The DNA was partially digested with the restriction enzyme FnuDII (a 4-base blunt end restriction enzyme with several cleavage sites in the nuclease gene) and mixed with DNA from plasmid pGV403, which had been digested with the restriction enzyme SmaI. The mixed DNA was ligated with T4 ligase and transformed to MT102. Selection was made on LA plates containing 20 μg/ml chloramphenicol (resistance of pGV403), and the transformants were replica plated onto DNase indicator plates. Twenty nuclease positive colonies were isolated and plasmid DNA prepared. The smallest plasmid had a 1.3 Kb DNA insertion, and the insert was mapped with respect to the EcoRV site as shown in FIG. **1**. This plasmid was denoted pGV403-SD2/10. A plasmid carrying the same insert but in the opposite orientation with respect to the unique EcoRI and HindIII recognition sites of pGV403 was denoted pGV403-SD2/14.

Nucleotide sequence of the nuclease gene

The method of Maxam and Gilbert was used (Proc. Natl. Acad. Sci USA 74, 1977, pp. 560–64), using the sequencing vector plasmid pGV403 (Amersham). The DNA to be sequenced is inserted into the SmaI site of the vector. The SmaI is flanked by two restriction sites for restriction enzyme Tth111I, which gives different 5-prime overhanging ends, and since the enzyme cleaves assymmetrically, the DNA can be sequenced directly after labelling with ^{32}P.

Therefore, the 1.3 Kb nuclease fragment originally cloned into the SmaI site of pGV403 was isolated from an agarose gel after digestion of the hybrid plasmid with Tth111I. The DNA fragment was digested with one of the restriction enzymes FnuDII or HaeIII and ligated to pGV403 DNA cleaved with SmaI and dephosphorylated. The DNA was then transformed to MT102, and selection was made on LA plates containing 20 μg/ml chloramphenicol. Plasmid DNA from the transformants was prepared and analysed. In this way, a series of pGV403 hybrid plasmids was constructed with insertion of DNA from 200–400 bp covering the whole 1.3 Kb fragment, and sequencing of these plasmids in both strands gave the nucleotide sequence shown above.

Analysis of the nucleotide sequence shown in FIG. **7** indicates that the nuclease is coded from position **386** to **1165**. Firstly, an open reading frame extends throughout this region which would encode a protein of 30,000 daltons. Secondly, a perfect ribosome binding site is present at position **374–78**, i.e. just upstream of the initiation codon. Thirdly, sequences which may constitute a regulatory region are present at position **330** to **336** ("−10 sequence") and position **306** to **313** ("−35 sequence").

To confirm that the nuclease is in fact encoded by the indicated sequence rather than from a long open reading frame present on the complementary strand, the inserts in pGV403-SD2/10 and pGV403-SD2/14 were excised by double digestion with EcoRI and HindIII. It should be noted that the orientations of the inserts are opposite relative to the two restriction sites of the pGV403 vector. The excised fragments were ligated to pPL195 which had been double digested with EcoRI and HindIII. The vector pPL195 is derived from pLc28 by inserting a polylinker containing EcoRI and HindIII recognition sites downstream from the λpL promoter. Following transformation into *E. coli* NF1 and selection at 30° C. for Ap^R, two plasmids were isolated, pPL195-SD2/10 and pPL195-SD2/14. In the former, the λpL promoter is located upstream of the putative nuclease coding region depicted above, while in the latter plasmid the λpL promoter is located in such a way that the complementary strand will be transcribed. *E. coli* NF1 is lysogenic for a defective λ coding for the temperature-sensitive λ repressor encoded by the c1857 gene. At 30° C. the cl repressor is active, and promoters regulated by the repressor such as λpL present on pPL195 are thus repressed. At temperatures above 37° C., the repressor is inactive and transcription from λpL in pPL195 will occur. When comparing the nuclease activity at 30° C. and 42° C., pPL195-SD2/10 but not pPL195-SD2/14 gave rise to temperature inducible nuclease synthesis indicating that the orientation of the nuclease-coding region relative to the λ promoter is correct in pPL195-SD2/10.

Furthermore, high levels of (temperature inducible) nuclease synthesis were obtained when the predicted nuclease coding region was joined directly to the λ promoter. An RsaI-HindIII fragment from pGV403-SD2/10 spanning the region from position **357** to **1295** (FIG. **7**) was ligated to pPL 195 digested with SmaI and HindIII whereby the coding region is positioned as in pPL195-SD2/10 relative to the λ promoter. This plasmid was denoted pPL195-SD2/Rl.

The nucleotide sequence corresponding to the amino terminus of the nuclease has been confirmed by amino acid sequence analysis of the partially purified protein. The nucleotide sequence corresponding to the carboxy terminus of the nuclease has been verified by nucleotide sequencing of the region using an alternative sequencing method, the dideoxy nucleotide sequencing of Sanger et al., *Proc. Nat. Acad. Sci. USA* 74, pp. 5463–5467.

The predicted amino terminal sequence of the nuclease indicates the presence of a signal peptide of 20 amino acids which is terminated by a recognition sequence for a signal peptidase at position **448**.

Enzyme activities of nuclease

Cultures of *Serratia marcescens* strain W225 and *E. coli* C600 harbouring the plasmid pBEU50-nuc+ were grown exponentially in LB medium at 30° C. At various times one ml samples were taken for determination of OD_{450} and nuclease activity. Nuclease activity was determined by adding 100 μl chlorophorm to release enzymes from the periplasm. After centrifugation at 10,000 r.p.m. for 15 min., 25 μl of the supernatant was taken for determination of nuclease activity. The sample containing nuclease was added to 0.5 ml of salmon sperm DNA (1 mg/ml) dissolved in 0.05M Tris (pH 8.0) +0.01M $MgCl_2$, and the mixture was incubated at 37° C. for one hour. Then 0.5 ml 4% PCA (perchloric acid) was added and left on ice for 30 min. The precipitate of undigested DNA was removed by centrifugation, and OD_{260} (absorption of UV light at wave-length 260 nm) was measured on a spectrophotometer in a quartz cuvette. The activities presented in Table 3 are OD_{260} values measured in this way from samples of the cultures growing into the stationary phase. It appears that in both cultures, the enzyme is preferentially synthesized in the late phase of the growth cycle.

TABLE 3

Strain	Nuclease activity: Cell Density (OD_{450})	Nuclease activity: Extracellular Nuclease Activity
C600/(pBEU50-nuc$^+$)	0.265	0
	0.448	0.005
	0.628	0.075
	0.800	0.135
	0.940	0.222
	1.28	0.447
	1.63	1.04
	2.50	1.78
	3.15	2.87
	4.20	3.90
	4.80	5.90
	5.10	7.70
	5.95	12.1
	6.80	14.2
	7.45	15.9
	8.08	35.2
W225	0.240	0
	0.386	0
	0.608	0
	0.865	0
	1.01	0
	1.48	0
	2.03	0
	2.56	0
	3.51	0.047
	4.30	0.555
	7.10	2.1
	9.50	4.6
	10.60	5.0
	11.4	6.7
	14.0	7.0
	14.7	7.1

In a parallel experiment, the distribution of nuclease between periplasm and growth medium was measured by dividing culture samples into two parts: One containing only cell-free growth medium, and the other containing material from both periplasm and growth medium (chlorophorm treatment as described above). The results are shown in Table 4.

TABLE 4

Strain	Nuclease Activity: Periplasm	Nuclease Activity: Growth Medium
Serratia marcescens W225	1.0	45.8
C600/pBEU50-nuc$^+$	1.5	1.23

As shown above, in *Serratia marcescens* W225 essentially all nuclease is totally excreted whereas only approx. 50% is excreted from *E. coli.*

EXAMPLE 2

Purification of nuclease

After 16–20 hours in the stationary growth phase, the fermentation medium from 25 liter cultures of *E. coli* MT102 containing plasmid pGV403-SD2 (described in Example 1) was harvested by ultrafiltration across a 0.45 μm membrane followed by concentration by ultrafiltration across a filter with a cut-off at 10,000 daltons. After dialysis against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, the preparation was filtered through a glass filter, and a 0.45 and 0.22 μm filter.

The enzyme preparation was tested for various parameters in a standard assay which is summarized as follows:

400 μl buffer comprising 50 mM Tris (pH 8.2), 1 mM $MgCl_2$, 50 μg/ml BSA, 100 ml DNA solution (5 mg/ml salmon sperm DNA in water) and 25 μl diluted enzyme preparation in the above buffer (without DNA) were incubated for 60 minutes at 37° C. To the reaction mixture were added 400 μl 4% cold perchloric acid. The reaction mixture was left standing on ice for 30 minutes and then centrifuged at 15,000×g for 5 minutes. The absorption was measured at 250 nm. 1 unit is defined as the activity which in the standard assay releases 1 OD_{260} of soluble material per ml of DNA in 1 hour.

To determine the pH-optimum, the pH of the standard assay buffer was varied and measured after the addition of DNA. The optimum range for nuclease activity is 7.5–9.6 with a maximum at pH 8.5–9.2. The Mg^{2+} optimum was determined by varying the concentration of $MgCl_2$ in the standard assay from 0 to 100 mM. There is a relatively clearly defined optimum in the range of 0.1–1 mM $MgCl_2$. However, the enzyme retained about 40% of its activity without addition of $MgCl_2$. The optimum concentration of monovalent cations was determined by varying the concentration of NaCl and KCl in the standard assay. The activity decreased rapidly at increasing Na^+ concentrations. The enzyme was shown to be active at 0–50 mM of KCl (there was no decrease in activity), which is important as cell lysis, especially of *E. coli*, yields a rather high quantity of K^+ as the intracellular concentration is 100–150 mM K^+. Short-term enzyme stability was determined by pre-incubating the enzyme in the standard assay buffer without any DNA at 4, 23 and 37° C. for 1, 4 and 18 hours, respectively. On addition of DNA, the enzyme activity was determined in the standard assay. Table 5 below shows the observed value for absorption at 250 nm in the standard assay. A stable enzyme will show the same values in each column.

TABLE 5

Preincubation	4° C.	23° C.	37° C.
1 hour	0.389	0.445	0.442
4 hours	0.415	0.485	0.403
18 hours	0.455	0.505	0.298

It appears from Table 5 that at 4° and 23° C., the enzyme is stable for 18 hours in the buffer. At 37° C. there is a decrease in activity on incubation for a longer period of time.

The effect of denaturing agents was determined by testing the activity of the enzyme in the presence of urea, non-ionic detergents (Brij ® 58, Triton ® X-100) and ionic detergents (SDS and sodium deoxy cholate). These substances were added to the preparation in the standard assay at different concentrations. The enzyme was found to be active in 1–8M urea, the enzyme actually showing an increased activity at 4–8M with a maximum at 4M urea. The enzyme was also fully active in the presence of non-ionic detergents such as Brij ® 58 (1%) and Triton ® X-100 (0.4%). With respect to ionic detergents, an SDS concentration of more than 0.01% leads to a complete inhibition of enzyme activity, while about 40% activity was retained in the presence of 1% sodium deoxy cholate.

The purity of the enzyme was analyzed by means of a standard denaturing SDS-PAGE. The enzyme preparation contained a number of protein bands. In the area corresponding to the apparent molecular weight of the nuclease (30,000), there was a distinct band estimated to represent 5–10% of the entire preparation.

Protease activity in the nuclease preparation was estimated by different assays. Firstly, 50 μl nuclease samples were spotted in water on protein (skim milk)

agar plates (20% milk in buffer). No formation of a clearing zone (degradation of the milk proteins on the plate) was observed after 24 hours at 37° C. and 48 hours at 23° C.

Secondly, no measurable degradation of azo-casein was observed on incubating 20 μl of the enzyme with 1 mg of azo-casein (buffer: 50 mM Tris (pH 8.0), 10 mM $MgCl_2$)) at 0, 16 and 30° C. for 12 hours, followed by measuring acid soluble azo dye at A_{370}. Thirdly, nuclease incubated at 37° C. in the presence of 5 mM $MgCl_2$ was analyzed by SDS-PAGE. No change in the pattern of the approximately 20 proteins present in the nuclease preparation, i.e. no autoproteolysis, was observed, indicating the absence of proteases. This means that in the practical application of the nuclease, a possible low content of proteolytic activity in the enzyme preparation will be minimal compared to the total content of protease in the cell lysate to be treated.

The ability of the nuclease to degrade DNA and RNA in the presence of organic solvents was determined. To aliquots of an FTL-lysate of *E. coli* MT 102 (1 part by volume of cells to 1 part by volume of Tris-EDTA buffer to which had been added 12,000 units of nuclease per ml prior to cell lysis, cf. Example 3 below) were further added phenol (1%), toluene (1%), chloroform (1%), ethanol (5%), or EDTA (0.25M). After incubation at 20° C. for 4.5 hours, the samples were analyzed by agarose gel electrophoresis. The sample to which EDTA had been added served as a control since the nuclease is virtually inactive at this concentration of EDTA. The addition of the various organic solvents did not affect the activity of the nuclease when compared to a sample to which no organic solvents had been added, and 95% of the DNA was degraded to fragments of 200 bp or less.

EXAMPLE 3

Reduction of viscosity in a cell lysate

The enzyme produced in Example 2 was added to a highly viscous FTL (lysozyme-freeze-thaw) lysate of 0.27 g *E. coli* at a total volume of 500 μl at about 2.6×10^2 and 2.6×10^3 units, respectively. To a series of samples, no Mg^{2+} had been added, while 10 mM Mg^{2+} had been added to another series of samples. The samples were incubated at 0° or 24° C.

Table 6 below shows the time at which the cell lysate was "aqueous", i.e. apparently having a viscosity approaching that of water (determined by aspirating a sample of the lysate into a pipette and observing whether the lysate runs out of the pipette as separate non-viscous drops).

TABLE 6

		Time after addition of enzyme (minutes)	
Temperature	Mg^{2+}	2.6×10^2 units	2.6×10^3 units
0° C.	–	72	12
	+10 mM	55	8
24° C.	–	55	5
	+10 mM	40	3

It should be noted, however, that considerable variations were observed in experiments involving different lysates. For instance, when FP (French Press) lysates were used, a certain degree of shearing of the nucleic acids was obtained. This type of lysate was found to provide a better substrate for the enzyme which is probably due to the less tightly packed gel structure of the FP lysate. The viscosity of an FP lysate (15 ml) obtained from 7.5 g of *E. coli* W3110 (wet weight) was reduced to "aqueous" on incubation of the lysate at 0° C. with 24 enzyme units/ml for 40 minutes.

Addition of nuclease prior to cell lysis

A. To 0.25 g of *E. coli* MC 1000 (wet weight) resuspended in 0.25 ml of TE (TE=10 mM Tris (pH 8.0), 1 mM EDTA) were added 12 units of the nuclease produced in Example 2. The suspension was subjected to FTL lysis according to standard procedures (3 cycles of freeze-thawing). The viscosity was monitored visually, the appearance of "aqueous" drops by pipetting being taken as an indication of a reduction of the viscosity of the lysate. After the last FTL cycle, the lysate was incubated at 0° C. After 5 minutes at 0° C., the lysate had become "aqueous".

This experiment unexpectedly showed the beneficial effect of adding the enzyme prior to cell rupture as only 24 enzyme units per ml are required to reduce the viscosity in 5 minutes if the nuclease is added before lysis compared to a requirement of 5200 units per ml of lysate in 12 minutes if the nuclease is added after lysis.

B. To obtain a better quantitation of the reduction of viscosity, this procedure was tested on *E. coli* lysates made by X-PRESS (Biotec) which combines freeze-thaw effects and high pressure lysis.

7.5 g of *E. coli* MC 1000 (wet weight) were resuspended in 7.5 ml of TE. $MgCl_2$ was added to 2 mM and nuclease to 25 units/ml. The suspension was frozen in the X-PRESS and subjected to five pressure cycles at −20° C. The homogenate was thawed at 0° C. over a period of 2 hours. Visually, the viscosity had been reduced at the time of thawing (i.e. "aqueous" drops by pipetting), but the extended period of thawing makes it difficult to establish a time zero prior to which the nuclease is not active. 24 units per ml of lysate are therefore useful to reduce the viscosity of X-PRESS lysates if the nuclease is added before lysis.

The homogenate was diluted to 37.5 ml with TE (0° C.), and the viscosity was monitored (Ostwald viscosimeter) for the next 24 hours. The nuclease digestion was continued in the viscosimeter which was incubated at 0° C. At the time indicated in FIG. 2 (abscissa), the viscosity was determined. The ordinate in FIG. 2 shows the observed viscosity relative to the viscosity of H_2O at 0° C. The reaction conditions were 9.6 units of nuclease per ml of lysate. The relative viscosity decreases rapidly during the first 10 minutes followed by a steady decrease during the subsequent hours of incubation at 0° C. At 24 hours the relative viscosity was 1.5.

C. 7.5 g of *E. coli* MC 1000 (wet weight) were resuspended in 7.5 ml of TE. $MgCl_2$ was added to 6 mM and nuclease to 24 units/ml. Bacteria plus enzyme was passed through a French Pressure Cell at 10,000 psi. The lysate was immediately incubated at 0° C. Time zero was taken as time of release from press. Upon release the lysate yielded "viscous" drops by pipetting which, however, changed to "aqueous" drops within 5 minutes of incubation at 0° C.

24 units of lysate are therefore also useful to reduce the viscosity of FP lysates.

At 5 minutes the lysate was diluted to 30 ml with TE (0° C.), and the viscosity was determined in the Ostwald viscosimeter at different times (FIG. 3, abscissa). The result is given as the relative viscosity (ordinate) using the viscosity of H_2O at 0° C. as reference. The reaction conditions in the viscosimeter were: 12 units of nuclease

per ml of lysate from 0.25 g of *E. coli* MC 1000 per ml, temperature = 0° C.

To illustrate the advantageous effects of adding the nuclease prior to lysis, the following experiment was carried out. Lysates were prepared as described above. To 15 ml samples of a suspension of *E. coli* W 3110 (7.5 g of cells) were added varying amounts of nuclease to final concentrations of 0.24 to 240 units per ml (lines **1** to **5** in FIG. **4**). Following lysis by French Press, the lysates were incubated at 0° C., and the viscosity was followed visually, i.e. by pipetting. The classification is depicted in FIG. **4**.

At 240 units of nuclease per ml (line **5**), the lysate was "aqueous" upon release from the press while the presence of nuclease at a concentration of 2.4 units per ml (line **2**) yielded "aqueous" drops after approximately 20 minutes at 0° C. At 0.24 units per ml (line **1**), the result at 70 minutes was "glycerol-like" drops which changed to "aqueous" drops during the subsequent 15 hours of incubation at 0° C.

The relative viscosity of 2.5 fold dilutions of the above samples was determined after 70 minutes and 15 hours of incubation at 0° C. Lines **2–5** show that in this experiment the visual impression "aqueous" drops spans a range of 1.5 to 2.1 in relative viscosity. With an excess of enzyme (line **5**), the minimum value obtainable is 1.5. This minimum is presumably reached at 70 minutes, indicating that the component of the viscosity ascribable to nucleic acids has been removed.

To provide information on the amount of nuclease needed in specific applications, the relationship between the amount of enzyme added and the viscosity at 70 minutes and at 15 hours was plotted (FIG. **5**). Addition of 3,600 units of nuclease yielded approximately the same value at 70 minutes and 15 hours of incubation at 0° C., namely 1.52 and 1.47, respectively. The value 1.50 may therefore be taken as the minimum value for the relative viscosity of the lysate in question.

After incubation for 70 minutes at 0° C., the relative viscosity is proportional to log(enzyme added) or log-(enzyme concentration). By extrapolation, the additon of 1,500 units (100 units per ml) would completely eliminate the viscosity component of the lysate that can be ascribed to the presence of nucleic acids, i.e. addition of enzyme in excess of 1,500 units or extension of the incubation period would yield no further reduction in the relative viscosity, the minimum value of which is 1.5.

It appears from the figure that a 10 fold reduction in the amount of enzyme added requires a 10 fold prolongation of incubation at 0° C. in order to achieve the same viscosity (e.g. 36 units/70 minutes vs 3.6 units/15 hours, 360 units/70 minutes vs. 36 units/15 hours.

To compare the new strategy of adding nuclease prior to cell rupture with the traditional method of adding nuclease after lysis of the cells, a 15 ml lysate was prepared as above but no nuclease was added prior to cell rupture. After French Press lysis, 360 units of nuclease were added to a final concentration of 24 units per ml, and the lysate was incubated at 0° C. Line **6** in FIG. **4** shows the stepwise elimination of visocity with the appearance of "aqueous" drops at 40 minutes. The relative viscosity at 70 minutes was comparable to that of the sample shown in line **3** (8 units per ml added before lysis), although the initial rate of reduction of viscosity is clearly different. It is estimated that the addition of approximately 1.5 units of nuclease per ml before cell lysis would yield a time pattern identical to that of line **6** but the resulting relative viscosity would clearly be higher, in the range of 2.13–2.53. The gain (in terms of enzyme requirement) may thus be either a factor of 3 or a factor of 20 depending upon the criteria used in defining "reduction of viscosity".

Samples taken from the lysates shown in FIG. **4**, lines **2–6**, when the "aqueous" state had just been reached, were subjected to agarose gel (1%) electrophoresis and subsequently stained with ethidium bromide. In all lanes, the stainable residual product constitutes a smear extending from a 21 kbp marker to the bromophenol blue band with decreasing amounts of slowly migrating material in the samples of higher nuclease concentration. This material comprises from less than 1 to a few percent of the nucleic acid present prior to the nuclease treatment (FIG. **6**).

EXAMPLE 4

Elimination of residual nucleic acids

From gel electrophoretic analyses of limit digests of bacterial lysates, it was concluded that about 0.1% of the nucleic acids present in a lysate is not available for the action of the nuclease. It is suggested that the presence of residual nucleic acids is ascribable to protective masking of specific sequences, perhaps a membrance associated area of the genome, as only a minimal fraction of the total amount of nucleic acid remains after treatment with the nuclease.

In order to remove the residual nucleic acids, cell lysates were treated with the nuclease in the presence of various protein denaturing agents.

FTL lysates of 0.25 g of *E. coli* (wet weight) in a total volume of 0.6 ml were treated with 240 units of nuclease in the presence of 1–12M urea. The lysates were incubated at 30° C. for 1 hour or 18 hours. After incubation, 5 μl of the residue were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

After 18 hours of digestion, a dramatically positive effect of 2–4M urea was observed, the presence of 4M urea in particular resulting in removal of all stainable material which has entered the gel.

FTL lysates of 0.68 g *E. coli* (wet weight) in a total volume of 2.5 ml TE were treated with 2.6×10^3 units of nuclease for 24 hours at 16° C. alone, in the presence of 0.1% SDS or 0.6% Triton ® X-100. Gel electrophoretic analyses indicated that residual nucleic acid could be digested by the nuclease if detergent was present.

From the results of these experiments, it appears that both types of detergents and protein denaturing agents make the residual, masked nucleic acids in a lysate available for the action of the nuclease.

EXAMPLE 5

Isolation of Serratia spp. A1

Bacteria were harvested from a rotten cucumber and plated out on DNase test agar. One colony showing a high level of exonuclease activity was further analyzed. Gram staining showed that it is gram negative. A preliminary identification indicated that the isolated organism is *Serratia liquefaciens*. However, until the classification is complete, it has tentatively been termed Serratia spp. A1 since there are many indications that it belongs to the Serratia group. The organism is resistant towards tetracylin and ampicillin, and it shows the same pattern of exoenzymes as the *Serratia marcescens*. (*Serratia liquefaciens* A1 was deposited in the DSM on May 8, 1985 under the Accession No. 3307.)

Preparation of chromosomal DNA from Serratia spp. A1

A culture of Serratia spp. A1 was grown overnight in LB medium and harvested by centrifugation (8,000 r.p.m. for 5 min.). The cells were washed twice in TEN-buffer (10 mM Tris, HCl, pH 8, 1 mM EDTA, 100 mM NaCl) and resuspended in 20 ml TEN-buffer containing 1 mg/ml lysozyme and 0.1 mg/ml RNase. The cells were incubated at 37° C. for 30 minutes and 20% SDS was added to a final concentration of 1%. After 60 minutes at a temperature of 37° C. (for total lysis), the lysate was incubated at 4° C. overnight. Next day the cell debris was removed by centrifugation (18,000 r.p.m. for 25 minutes). The supernatant was transferred to a new tube containing 2 ml 3M sodium acetate and 2 volumes of isopropanol. Upon gentle mixing, the DNA precipitated in threads which were picked up by means of a curved glass needle. The precipitated DNA was washed twice in 80% ethanol and resuspended in TEN-buffer. The DNA was further purified by buoyant density gradient centrifugation, and after appropriate dilution it was extracted with phenol and dialysed against TE-buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). Finally, the DNA was tested for absence of nuclease by incubation at 37° C. with restriction enzyme buffer.

Construction of a gene bank from Serratia spp. A1

The cloning vector plasmid pNU121 was used for the construction of a gene bank from Serratia spp. A1. The plasmid is described in Example 1.

pNU121 DNA with a unique EcoRI site in the C_I gene was digested with the restriction enzyme EcoRI and mixed with Serratia spp. A1 DNA partially digested with EcoRI. The DNA was ligated at 15° C. overnight with T4 DNA ligase and transformed to *E. coli* strain MT102. Selection was made at 37° C. on LB plates containing 8 μg/ml tetracyclin, so that only cells harbouring pNU121 with inserted DNA will give rise to colonies. Approximately 8,000 colonies representing a gene bank of Serratia spp. A1 were isolated by this procedure.

Screening for lipase activity

E. coli MT102 cells were transformed with the genomic bank of Serratia spp. A1 and cells carrying hybrid plasmids selected on LB plates with tetracyclin. Colonies were picked and transferred to microtiter dishes, each well containing A+B medium+1% casamino acids+thiamin and 200 mg/ml streptomycin and 8 μg/ml tetracyclin. Cells were grown overnight at 37° C. and replicas were made of the dishes. The substrate for the lipase enzyme, p-nitrophenylpalmitate was first suspended in isopropanol at a concentration of 6 mg/ml. 10 ml of the suspension was added to 90 ml 0.05M phosphate buffer pH 8.0 containing 207 mg of sodium deoxycholate. 0.5 ml of this solution was added to each well in the dishes. Yellow colour in a well indicates presence of lipase activity. One such clone was obtained. DNA was prepared and used to transform *E. coli* CSH50. Transformants were lipase positive. One such clone was isolated and DNA was prepared. The selected clone did not exhibit protease, phospholipase or nuclease activity.

The lipase-carrying plasmid pNU121-lip+

Plasmid DNA isolated from the lipase positive clone consisted of pNU121 with an inserted EcoRI fragment of approximately 8.4 Kb. The hybrid plamid is denoted pNU121-lip+. (*Escherichia coli* CSH50/pNU121-lip+ was deposited in the DSM on May 8, 1985 under the Accession No. 3313.).

Enzyme activities of lipase

The action of lipase activity on the substrate p-nitrophenylpalmitate can be followed spectophotometrically at OD_{410}. When both *E. coli*/pNU121-lip+ and Serratia spp. A1 were grown exponentially in A+B medium+1% casamino acids and thiamin, the enzyme was shown to be present in the culture medium.

EXAMPLE 6

Preparation of chromosomal DNA from Serratia spp. A1

A culture of Serratia spp. A1 (vide Example 5) was grown overnight in LB medium and harvested by centrifugation (8,000 r.p.m. for 5 minutes). The cells were washed twice in TEN-buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 100 mM NaCl) and resuspended in 20 ml TEN-buffer containing 1 mg/ml lysozyme and 0.1 mg/ml RNase. The cells were incubated at 37° C. for 30 minutes and 20% SDS was added to a final concentration of 1%. After 60 minutes at a temperature of 37° C. (for total lysis), the lysate was incubated at 4° C. overnight. Next day the cell debris was removed by centrifugation (18,000 r.p.m. for 25 minutes). The supernatant was transferred to a new tube containing 2 ml 3M sodium acetate and 2 volumes of isopropanol. Upon gentle mixing, the DNA precipitated in threads which were picked up by means of a curved glass needle. The precipitated DNA was washed twice in 80% ethanol and resuspended in TEN-buffer. The DNA was further purified by buoyant density gradient centrifugation, and after appropriate dilution it was extracted with phenol and dialysed against TE-buffer (10 mM Tris, HCl pH 8, 1 mM EDTA). Finally, the DNA was tested for absence of nuclease by incubation at 37° C. with restriction enzyme buffer.

Construction of a gene bank from Serratia spp. A1

The cloning vector plasmid, pNU121 (cf. Example 1), was used for the construction of a gene bank from Serratia spp. A1 (cf. Example 5).

pNU121 DNA with a unique EcoRI site in the C_I gene was digested with the restriction enzyme EcoRI and mixed with Serratia spp. A1 DNA partially digested with EcoRI. The DNA was ligated at 15° C. overnight with T4 DNA ligase and transformed to *E. coli* strain MT102. Selection was made at 37° C. on LB plates containing 8 μg/ml tetracyclin, so that only cells harbouring pNU121 with inserted DNA gave rise to colonies. Approximately 8,000 colonies representing a gene bank of Serratia spp. A1 were isolated by this procedure.

Screening for phospholipase-positive clones

E. coli MT102 cells were transformed with the genomic bank of Serratia spp. A1 and cells carrying hybrid plasmids selected on LB plates with tetracyclin colonies were replicated on egg yolk plates with tetracycline. A clearing zone around and a white precipitation on top of a colony indicates phospholipase activity. Fifteen such colonies were isolated, DNA was prepared and used to tranform CSH50. The phospholipase clone used was such a clone. The selected clone pNU121-phl+ exhibited only the phospholipase activity. (*Escherichia coli*

MT102/pNU121-phl+ was deposited in the DSM on May 8, 1985 under the Accession No. 3311.)

The phospholipase carrying plasmids pNU121-phl+ and pOU57-phl+

The plasmid DNA isolated from the phospholipase-producing clone, pNU121-phl+, consisted of pNU121 with a 3.2 Kb EcoRI fragment inserted in the C_I gene (FIG. **8**). This EcoRI fragment was cloned in the runaway plasmid pOU57. This runaway hybrid plasmid pOU57-phl+ conferred the phospholipase phenotype to other *E. coli* strains, and elevated expression of phospholipase was observed when present in Serratia strains. (*Escherichia coli* S17-1/pOU57-phl+ was deposited in the DSM on May 8, 1985 under the Accession No. 3312.) The phospholipase expression was amplified upon a temperature increase from 30° C. to 40° C. in the tested strains.

Enzyme activities of phospholipase

When *E. coli* cells containing the plasmid pNU121-phl+ were grown in A+B+1% casamino acids and thiamin, or LB, the phospholipase is only detected in the culture medium after the culture has reached a cell density corresponding to 0.7 OD_{450} units. The viability of the *E. coli* strain was by no means affected by the presence of the plasmid.

The assay for phospholipase activity is based on reaction with egg yolk. Activity is assayed in 2% agarose gels containing egg yolk and chloramphenicol which inhibits growth of cells and protein synthesis in the gels.

SmaII wells were made in the gel into which 5 μl samples of supernatant (cells having been removed by centrifugation) of the growing culture were pipetted.

The enzyme reaction with the egg yolk produced a clearing zone in the turbid gel. Enzyme diffusion speed, i.e. mm^2 clearing zone per unit time, is used as a measure of enzyme activity. Measurements of phospholipase activity from growing cultures of *E. coli*/pNU121-phl+ and Serratia spp. A1 are shown in Table 7.

TABLE 7

	Activities of Phospholipase	
Culture	Cell Density OD_{450}	Extracellular Phospholipase Activity: mm^2/hour
MT102/pNU121-phl+	0.1	0
	0.5	0
	0.7	0.4
	0.9	0.7
	1.0	0.9
	1.3	1.3
	1.4	1.5
	1.5	1.8
Serratia spp. A1	0.1	0
	0.5	0
	0.7	0.02
	0.9	0.10
	1.2	0.35
	1.8	0.90
	2.0	1.1

It appears that the *E. coli* culture secretes the enzyme to the culture medium more efficiently than Serratia. In both strains, the appearance of enzyme in the media is in the late exponential growth phase and continues into the stationary phase. Presence of 1% glucose in the media efficiently blocks synthesis of the enzyme in both hosts (not shown). A minor amount of detergent in the culture media (0.5% Tween® 80) has a stimulating effect on secretion (not shown).

DNA sequencing of the phospholipase clone

The 3.2 Kb EcoRI restriction fragment containing the phospholipase gene was sequenced using the "shotgun" cloning method of Messing et al. (*Nucl. Acid Res.* 9, 1981, p. 309) on the M13 phage derivatives Mp8 and Mp9 and the dideoxy chain thermination technique of Sanger et al. (*Proc. Natl. Acad. Sci. USA* 74, 1981, p. 5463). In subcloning of the fragment, many different restriction enzymes were used: Sau3A, TaqI, AluI, RsaI, SalI, SmaI, PstI, EcoRI, PvuI, BssHII and EcoRV. The entire sequence was established by merging the collection of small (100–300 bases) pieces of DNA sequence. Most of the sequence has been determined for both strands.

The sequence (FIG. **9**) shows a major reading frame which starts from the left end of the fragment, at position **416**, passing the SalI site to position **1372** where it ends.

Upstream of the frame is a Shine-Dalgarno homology (Shine, Dalgarno, *Nature* 254, 1975, p. 34) AAGGAG at position **405** immediately upstream of the ATG start codon. Upstream of the reading frame is a promoter region consisting of a −35 sequence CTGCC at position **351** and a −10 sequence TATTTA at position **374**. Upstream of the −35 sequence is a potential CAP-binding site from position **306** to **336**.

The sequence indicates the presence of a gene which encodes a 319 amino acids protein with a predicted molecular weight of 34,056 daltons.

Insertion of the DNA fragment from position **0** to the PstI site at **441** upstream of the lac genes indicated the presence of a functional promoter in this DNA fragment. This promoter initiates lac expression at an OD_{450} of 0.7 in a growing population of cells. Also this promoter was non-functional at any cell density in the presence of glucose, indicating catabolite repression probably mediated via the indicated CAP binding site.

By subcloning it has been verified that the necessary genetic information for the extracellular phospholipase activity is located within the 1.2 Kb fragment from position **360** to the FspI site at position **1551**. It was also found that, in keeping with sequence information, it was necessary to clone this fragment in front of a promoter in order to obtain phospholipase activity in *E. coli* cells. In this way, orientation of the gene was also verified. The direction of transcription of the gene is from the left EcoRI site to the FspI site in keeping with sequence data. The promoter used was the temperature inducible system of c1857 and λpR. At 30° C. synthesis of phospholipase in *E. coli* cells was very low as judged from the normal plate assay. At temperatures above 37° C., there was a large production of enzyme. The gene product of this 1.2 Kb DNA fragment has been identified both in vivo and in vitro by incorporating radioactively labelled methionine. By SDS-polyacrylamide gel electrophoresis, the size of the gene product has been determined to 34 Kdalton, and it has been shown that in this gel system, phospholipase activity comigrates with the radioactively labelled 34 Kdalton protein.

We claim:

1. A method of producing an extracellular hydrolytic Serratia spp. enzyme selected from the group consisting of exocellular nuclease, exocellular phospholipase and exocellular lipase, comprising cultivating, *E. coli* in a culture medium, said *E. coli* harboring a hybrid plasmid

which comprises DNA encoding an extracellular hydrolytic Serratia spp. enzyme selected from the group consisting of exocellular nuclease, exocellular phospholipase and exocellular lipase, operably linked to a promotor functional in said *E. coli*, under conditions conductive to the expression of said enzyme and its secretion into the culture medium, and harvesting the enzyme from the culture medium.

2. A method according to claim **1** wherein the Serratia spp. enzyme is a Serratia spp. nuclease.

3. A method according to claim **1** wherein the Serratia spp. enzyme is a Serratia spp. phospholipase.

4. The method of claim **1** wherein the hybrid plasmid is a plasmid with a conditionally uncontrolled replication behavior.

5. The method of claim **1** wherein the DNA sequence is

```
1                                                  50                                                 100
GCCCCGCTGAAAGTCGTCTTGAAGGTGCTGCTGTCTTCGGTGGACAAGATAGTCAAAGTGCAGATCGCCAACAAGGTCGAGCAGGAAATCGCCCAGATCG
CGGGGCGACTTTCAGCAGAACTTCCACGACAGAAGCCACCTGTTCTATCAGTTTCACGTCTAGCGGTTGTTCCAGCTCGTCCTTTAGCGGGTCTAGC

101                                                150                                                200
AACAGCGGTGGTGCGCCGCCGGCGGCGTCCGGTATCACCGGCGATCTGGCGCAGATGACGCATTACGTTCGCCGCACGGTCAACGACCTGCTGGATAATC
TTGTCGCCACCACGCGGCGGCCGCCGCAGGCCATAGTGGCCGCTAGACCGCGTCTACTGCGTAATGCAAGCGGCGTGCCAGTTGCTGGACGACCTATTAG

201                                                250                                                300
CCCCCTTTGAAACGGCGTCTGTTGGACGGCTTTATTTCCGCCGCATTTCACGTGCGGCGTGGCTGTACCATGACTGACACATTCACACAACATGAATATGTT
GGGGGAAACTTTGCCGCAGACAACCTGCCGAAATAAAGGCGGCGTAAAGTGCACGCCGCACCGACATGGTACTGACTGTGTAAGTGTTGTACTTATACAA

301                                                350                                                400
GCATTGTTGTATTCGTTTCACTGCGATAAGTTTAATTTACTGTAAATATATACAGTACTTTTTTTTAACTTATTGAGGATATGAATATGCGCTTTAACAAC
CGTAACAACATAAGCAAAGTGACGCTATTCAAATTAAATGACATTTATATATGTCATGAAAAAAATTGAATAACTCCTATACTTATACGCGAAATTGTTG

401                                                450                                                500
AAGATGTTGGCCTTGGTCGCCCTGCTGTTCGCCGCACAGGCGTCGGCCGACACGCTCGAATCCATCGACAACTGCGCGGTCGGCTGCCCGACCGGCGGCA
TTCTACAACCGGAACCAGCGGGACGACAAGCGGCGTGTCCGCAGCCGGCTGTGCGAGCTTAGGTAGCTGTTGACGCGCCAGCCGACGGGCTGGCCGCCGT

501                                                550                                                600
GCAGCAACGTGTCTATCGTGCGTCATGCTTATACGTTGAACAACAACAGCACCACCAAGTTCGCCAACTGGGTGGCTTATCACATCACCAAAGACACACC
CGTCGTTGCACAGATAGCACGCAGTACGAATATGCAACTTGTTGTCGTGGTGGTTCAAGCGGTTGACCCACCGAATAGTGTAGTGGTTTCTGTGTGG

601                                                650                                                700
GGCCAGCGGCAAGACGCGCAACTGGAAAACCGATCCGGCGCTCAACCCGGCGGACACGTTGGCGCCCGCCGATTACACTGGCGCCAACGCGGCGCTGAAG
CCGGTCGCCGTTCTGCGCGTTGACCTTTTGGCTAGGCCGCGAGTTGGGCCGCCTGTGCAACCGCGGGCGGCTAATGTGACCGCGGTTGCGCCGCGACTTC

701                                                750                                                800
GTCGATCGCGGTCATCAGGCGCCGCTGGCCTCGCTGGCGGGCGTCTCCGACTGGGAATCGCTGAATTACCTGTCCAACATCACGCCGCAAAAGTCCGATC
CAGCTAGCGCCAGTAGTCCGCGGCGACCGGAGCGACCCGCCCGCAGAGGCTGACCCTTAGCGACTTAATGGACAGGTTGTAGTGCGGCGTTTTCAGGCTAG

801                                                850                                                900
TTAACCAGGGCGCCTGGGCGCGGCTGGAAGATCAGGAACGCAAGCTGATCGATCGGCGCCGATATCTCCTCGGTCTATACCGTGACCGGGCCGCTGTATGA
AATTGGTCCCGCGGACCCGCGACCTTCTAGTCCTTGCGTTCGACTAGCGCGGCTATAGAGGAGCCAGATATGGCACTGGCCCGGCGACATACT

901                                                950                                                1000
ACGCGATATGGGCAAACTGCCGGGCACCCAGAAAGCGCACACCATCCCCCAGCGCCTACTGGAAGGTGATTTTCATCAACAACAGCCCGGCGGTGAACCAC
TGCGCTATACCCGTTTGACGGCCCGTGTGGGTCTTTCGCGTGTGGTAGGGGTCGCGGATGACCTTCCACTAAAAGTAGTTGTTGTCGGGCCGCCACTTGGTG

1001                                               1050                                               1100
TATGCCGCTTTCCTGTTCGATCAGAACACGCCGAAGGGCGCCGATTTCTGCCAATTCCGCGTGACGGTGGACGAGATCGAGAAACGCACCGGCCTGATCA
ATACGGCGAAAGGACAAGCTAGTCTTGTGCGGCTTCCCGCGGCTAAAGACGGTTAAGGCGCACTGCCACCTGCTCTAGCTCTTTGCGTGGCCGGACTAGT
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1101                                                1150
  TCTGGGCCGGTCTGCCGGACGTGCAGGCTTCGCTGAAGAGCAAACCGGCGTCCTGCCGGAGTTGATGGGCTGCAAAAACTGACGAAAACCGCCGAAG
  AGACCCGGCCAGACGGCCTGCTGCACGTCCGAAGCGACTTCTCGTTTGGCCGCAGGACGGCCTCAACTACCCGACGTTTTTGACTGCTTTTGGCGGCTTC

1201                                                1250                                                1294
  CGGGTTTATTTTTCACGCGGGCGCCGGCGGATTATCCCGTCGCGCCTTTTGCGCGGCGGCCAACTCACGCTGACGGGGTGAGGCTACCGGGGCC
  GCCCAAATAAAAAGTGCGCCCGCGGCCGCCTAATAGGGCAGCGCGCCGGAAAACGCGCCGCCGGTTGAGTGCGACTGCCCCACTCCGATGGCCCCGG
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6. A method according to claim 1 of producing the enzyme substantially free from other bacterial protein, in which the enzyme is secreted from the *E. coli* into the culture medium and is harvested from the culture medium.

7. The method of claim 1 wherein the DNA encodes a Serratia spp. nuclease which, prior to removal of the amino-terminal signal peptide, has the amino acid sequence

MetArgPheAsnAsnLysMetLeuAlaLeuValAlaLeuLeuPheAlaAlaGlnAlaSerAlaAsp
ThrLeuGluSerILeAspAsnCysAlaValGlycysProThrGlyGlySerSerAsnValSerIleValArg
HisAlaTyrThrLeuAsnAsnAsnSerThrThrLysPheAlaAsnTrpValAlaTyrHisIleThrLysAsp
ThrProAlaSerGlyLysThrArgAsnTrpLysThrAspProAlaLeuAsnProAlaAspThrLeuAlaProAla
AspTyrThrGlyAlaAsnAlaAlaLeuLysValAspArgGlyHisGlnAlaProLeuAlaSerLeuAlaGly
ValSerAspTrpGluSerLeuAsnTyrLeuSerAsnIleThrProGlnLysSerAspLeuAsnGlnGlyAla
TrpAlaArgLeuGluAspGlnGluArgLysLeuIleAspArgAlaAspIleSerSerValTyrThrValThr
GlyProLeuTyrGluArgAspMetGlyLysLeuProGlyThrGlnLysAlaHisThrIleProSerAlaTyr
TrpLysValIlePheIleAsnAsnSerProAlaValAsnHisTyrAlaAlaPheLeuPheAspGlnAsnThr
ProLysGlyAlaAspPheCysGlnPheArgValThrValAspGluIleGluLysArgThrGlyLeuIleIle

TrpAlaGlyLeuProAspAspValGlnAlaSerLeuLysSerLysProAlaSerCysArgSer ┆.

8. The method of claim 1 wherein the DNA encodes a Serratia spp. phospholipase encoded by the DNA sequence

ATGAGTATGCCTTTAAGTTTTACCTCTGCAGTATCCCCGGTGGCCGCGATCCCTACGCCTCGCGCCGCTGCCGAGACGCGGACGG
TACTCATACGGAAATTCAAAATGGAGACGTCATAGGGGCCACCGGCGCTAGGGATGCCCGAGCGCGGCGACGGCTCTGCGCCTGCC

CGGCGAGCCTGCGGCACGCCGGCAAATCCGGGCCGGTGGCCTCTCCCTCTCAGAACACGCTGAACGCGCAGAATCTGTTGAATAC
GCCGCTCGGACGCCGTGCGGCCGTTTAGGCCCGGCCACCGGAGAGGGAGAGTCTTGTGCGACTTGCGCGTCTTAGACAACTTATG

GCTGGTCGGCGATATCTCAGCGGCGGCACCGACGGCGGCGCAGCGCCGGGCGTGACGCGGGGGCAGCAATCGCAGGAGGGGGAT
CGACCAGCCGCTATAGAGTCGCCGCCGTGGCTGCCGCGCCGCCGTCGCGGCCCCCGTCGTTAGCGTCCTCCCCCCTA

TATGCGTTGGCGCTGTTGGCCAAGGACGTTTACTCACTCAATGGCCAGGGCGCCGCCGGGTTCAACCGCCTGAGCGACACCGCTG
ATACGCAACCGCGACACAACCGGTTCCTGCAAATGAGTGAGTTACCGGTCCCGCGGCGGCCCAAGTTGGCCGGACTCGCTGTGGCGAC

CTCGGTTTCGGCATCGATCCCGCCAGCCTGCACGACGCGGGCAGCGGTTTCCCAGGCTGGGATTTACAGCAACGACAAACAGTAT
GAGCCAAAGCCGTAGCTAGGGCGGTCGGACGTGCTGCGCCCGTCGCCAAAGGGTCCGACCCTAAATGTCGTTGCTGTTTGTCATA

GTGTTGGCGTTCGCCGGCACCAACGACTGGCCGCGATTGGCTGAGCAACGTGCGGCAGGCGACGGGCTATGACGATGTGCAGTAC
CACAACCGCAAGCGGCCGTGGTTGCTGACCGGCGCTAACCGACTCGTTGCACGCCGTCCGCTGCCCGATACTGCTACACGTCATG

AATCAGGCGGTTGCCGCTGCCAAAAGCCGCCAAGGCGGCCTTCGGCGATGCGCTGGTGATCGCCGGCCATTCGCTTGGCGGTGGT
TTAGTCCGCCAACGGCGACGGTTTTCGGCGGTTCCGCCGGAAGCCGCTACGCGACCACTAGCGGCCGGTAAGCGAACCGCCACCA

CTGGCGGCCACCGCCGCGCTGGCGACCGGCACCGTCGCGGTCACCTTCAACGCGGCCGGGGTCTCGGATTACACCCTGAATCGCCT
GACCGCCGGTGGCGGCGCGACCGCTGGCCGTGGCAGCGCCAGTGGAAGTTGCGCCGGCCCCCAGAGCCTAATGTGGGACTTAGCGGA

GGGCATCGATCCGGCGGCAGCGAAGAAAGATGCCGAAGCCGGCGGCATTCGCCGTACAGCGAGCAATATGACATGCTGACCAGCA
CCCGTAGCTAGGCCGCGTCGCTTCTTTCTACGGCTTCGGCCGCCGTAAGCGGCATGTCGCTCGTTATACTGTACGACTGGTCGT

CCCAGGAGTCGACCTCGCTGATCCCGGATGCCATCGGCCACAACATCACCCTGGCCAACAACGATACCCTGACCGGCATCGATGA
GGGTCCTCAGCTGGAGCGACTAGGGCCTACGGTAGCCGGTTGTTGTAGTGGGACCGGTTGCTATGGGACTGGCCGTAGCTACT

CTGGCGGCCGAGCAAACATCTGGATCGCAGCCTGACGGCGCACGGCATCGACAAGGTGATAAGCTCGATGGCGGAACAAAAGCCG
GACCGCCGGCTCGTTTGTAGACCTAGCGTCGGACTGCCGCGTAGCTGTTCCACTATTCGAGCTACCGCCTTGTTTTTCGGC

TGGGAGGCGAAGGCCAATGCCTGA
ACCCTCCGCTTCCGGTTACGGACT.

9. A DNA fragment comprising a DNA sequence encoding a Serratia spp. phospholipase.

10. A DNA fragment according to claim 9 wherein the DNA sequence is

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201                                        250                                        300
GTTATCGCCCGCACCTTTACCGAAAGCCTGTAATTTGCGGCGCAGTCAATCAGGAGCTTCGGCTCCCTTTCTGGCGTTTGGCGGCCGAAAACCGAACGTG
CAATAGCGGGCGTGGAAATGGCTTTCGGACATTAAACGCCGCGTCAGTTAGTCCTCGAAGCCGAGGGAAAGACCGCAAACCGCCGGCTTTTGGCTTGCAC

                 CAP-SITE, PROMOTER AREA.
301                                        350                                        400
GATCACATTCTGTACAAAGATAAGCATTTCTAATACAGAACTCATCCGACCTGCCGATAGCTAAATCAGCACCTATTTAGGTGCTCAATAAAAAGTCTAT
CTAGTGTAAGACATGTTTCTATTCGTAAAGATTATGTCTTGAGTAGGCTGGACGGCTATCGATTTAGTCGTGGATAAATCCACGAGTTATTTTTCAGATA

401  S.D.         phl-gene------------->       PstI     450                                        500
CGACAAGGAGTCGGCATGAGTATGCCTTTAAGTTTTACCTCTGCAGTATCCCCGGTGGCCGCGATCCCTACGCCTCGCGCCGCTGCCGAGACGCGGACGG
GCTGTTCCTCAGCCGTACTCATACGGAAATTCAAAATGGAGACGTCATAGGGGCCACCGGCGCTAGGGATGCGGAGCGCGGCGACGGCTCTGCGCCTGCC
 . . . . . . fMET Ser METPro Leu Ser PHE THR SER ALA VALSER PRO VALALA ALA ILE Pro Thr Pro ARGALA ALA ALA Glu Thr arg Thr A.

                                                                                          EcoRV
501                                        550
CGGCGAGCCTGCGGCACGCCGGCAAATCCGGGCCGGTGGCCTCTCCCTCTCAGAACACGCTGAACGCGCAGAATCTGTTGAATACGCTGGTCGGCGATAT
GCCGCTCGGACGCCGTGCGGCCGTTTAGGCCCGGCCACCGGAGAGGGAGAGTCTTGTGCGACTTGCGCGTCTTAGACAACTTATGCGACCAGCCGCTATA
LAALA Ser LEU arg His ALAGLY LYS SER gly PRO VALALA SER Pro SER GLNASN Thr LEU ASN ALA GLN Asn LEU Leu Asn Thr LEU Val GLY ASP IL

601                                        650                                        700
CTCAGCGGCGGCACCGACGGCGGCGGCAGCGCCGGGCGTGACGCGGGGGCAGCAATCGCAGGAGGGGGATTATGCGTTGGCGCTGTTGGCCAAGGACGTT
GAGTCGCCGCCGTGGCTGCCGCCGCCGTCGCGGCCCGCACTGCGCCCCCGTCGTTAGCGTCCTCCCCCTAATACGCAACCGCGACAACCGGTTCCTGCAA
ESer ALA ALA ALA PRO Thr ALA ALA ALA ALA PRO GLY VAL Thr arg gly GLNGln Ser GLNGlu gly ASP TYR ALA Leu ALA LEU Leu ALA Lys ASP Val

701                                        750                                        800
TACTCACTCAATGGCCAGGGCGCCGCCGGGTTCAACCGCCTGAGCGACAGCGCGCTGCTCGGTTTCGGCATCGATCCCGCCAGCCTGCACGACGCGGGCA
ATGAGTGAGTTACCGGTCCCGCGGCGGCCCAAGTTGGCGGACTCGCTGTCGCGCGACGAGCCATAGCCGTAGCTAGGGCGGTCGGACGTGCTGCGCCCGT
TYR Ser Leu Asn GLY GLNGLY ALA ALA gly PHE ASN ARG LEU Ser ASP Ser ALA LEU Leu GLY PHE GLY ILE ASP Pro ALA Ser LEU His ASP ALA GLY S

801                                        850                                        900
GCGGTTTCCAGGCTGGGATTTACAGCAACGACAAACAGTATGTGTTGGCGTTCGCCGGCACCAACGACTGGCGCGATTGGCTGAGCAACGTGCGGCAGGC
CGCCAAAGGTCCGACCCTAAATGTCGTTGCTGTTTGTCATACACAACCGCAGGCGGCCGTGGTTGCTGACCGCGCTAACCGACTCGTTGCACGCCGTCCG
er GLY PHE GLNALA gly ILE TYR Ser ASN ASP LYS GLNTYR VAL Leu ALA PHE ALA GLY THR ASN ASP TRP ARG ASP TRP LEU Ser ASN VAL arg GLNAL

901                                        950                                        1000
GACGGGCTATGACGATGTGCAGTACAATCAGGCGGTTGCCGCTGCCAAAAGCCGCCAAGGCGGCCTTCGGCGATGCGCTGGTGATCGCCGGCCATTCGCT
CTGCCCGATACTGCTACACGTCATGTTAGTCCGCCAACGGCGACGGTTTTCGGCGGTTCCGCCGGAAGCCGCTACGCGACCACTAGCGGCCGGTAAGCGA
AThr GLY TYR ASP ASP VALGLNTYR Asn GLNALA VALALA ALA ALA LYS Ser ARGGln GLY GLY Leu arg arg CYS ALAGLY ASP ARGarg Pro PHE ALA

1001                                       1050                                       1100
TGGCGGTGGTCTGGCGGCCACCGCCGCGCTGGCGACCGGCACCGTCGCGGTCACCTTCAACGCGGCCGGGGTCTCGGATTACACCCTGAATCGCCTGGGC
ACCGCCACCAGACCGCCGGTGGCGGCGCGACCGCTGGCCGTGGCAGCGCCAGTGGAAGTTGCGCCGGCCCCAGAGCCTAATGTGGGACTTAGCGGACCCG
TRP arg TRP SER GLY GLY His ARGARG ALA GLY ASP arg His ARGARGGLY His Leu Gln ARGGLY arg GLY Leu gly Leu His Pro GLU Ser Pro gly H
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1101      .         .         .         .        1150        .         .         .      SalI        1200
ATCGATCCGGCGGCAGCGAAGAAAGATGCCGAAGCCGGCGGCATTCGCCGTACAGCGAGCAATATGACATGCTGACCAGCACCCAGGAGTCGACCTCGCT
TAGCTAGGCCGCCGTCGCTTCTTTCTACGGCTTCGGCCGCCGTAAGCGGCATGTCGCTCGTTATACTGTACGACTGGTCGTGGGTCCTCAGCTGGAGCGA
IS arg SER GLY GLY Ser GLU GLU arg CYS arg Ser arg arg HIS Ser PRO TYR Ser Glu Gln TYR ASP MET LEU THR Ser THR GLN Glu Ser THR Ser LE

1201      .         .         .         .        1250        .         .         .         .        1300
GATCCCGGATGCCATCGGCCACAACATCACCCTGGCCAACAACGATACCCTGACCGGCATCGATGACTGGCGGCCGAGCAAACATCTGGATCGCAGCCTG
CTAGGGCCTACGGTAGCCGGTGTTGTAGTGGGACCGGTTGTTGCTATGGGACTGGCCGTAGCTACTGACCGCCGGCTCGTTTGTAGACCTAGCGTCGGAC
UILE PRO ASP ALA ILE GLY His ASN ILE THR LEU ALA ASN ASN ASP THR LEU THR GLY ILE ASP ASP TRP arg PRO Ser LYS HIS LEU ASP ARG Ser LEU

1301      .         .         .         .        1350  phl-stop      .         .         .        1400
ACGGCGCACGGCATCGACAAGGTGATAAGCTCGATGGCGGAACAAAAGCCGTGGGAGGCGAAGGCCAATGCCTGAAGGGCGTCGCTTGCGGCGGGCGCTG
TGCCGCGTGCCGTAGCTGTTCCACTATTCGAGCTACCGCCTTGTTTTCGGCACCCTCCGCTTCCGGTTACGGACTTCCCGCAGCGAACGCCGCCCGCGAC
Thr ALA His GLY ILE ASP Lys VAL ile Ser Ser MET ALA GLU Gln Lys PRO TRP Glu ALA Lys ALA Asn ALA.

1401      .         .         .         .        1450        .         .         .         .        1500
GCGATAGCCTTACTGGCGCTGGTCGCGGTGACCGGTTTACTGATGATGGCTAAGGAGCAACAGATGGGGCAGGAGATTTCGCCGTTTGACGGCCACAGCG
CGCTATCGGAATGACCGCGACCAGCGCCACTGGCCAAATGACTACTACCGATTCCTCGTTGTCTACCCCGTCCTCTAAAGCGGCAAACTGCCGGTGTCGC

1501      .         .         .         .        1550 FspI      .         .         .         .        1600
AATCTGGCGCTGGCTCAGGCGGTGGCGCGTGGCGATACGCAGGGTATCCATGCGCAGGCCACGCAGGATCGCTTGCGCGAACGGGGCGATCGGCAGGTCA
TTAGACCGCGACCGAGTCCGCCACCGCGCACCGCTATGCGTCCCATAGGTACGCGTCCGGTGCGTCCTAGCGAACGCGCTTGCCCCGCTAGCCGTCCAGT.
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US005219727A

United States Patent [19]

Wang et al.

[11] Patent Number: 5,219,727

[45] Date of Patent: Jun. 15, 1993

[54] **QUANTITATION OF NUCLEIC ACIDS USING THE POLYMERASE CHAIN REACTION**

[75] Inventors: **Alice M. Wang**, Walnut Creek; **Michael V. Doyle**, Oakland, both of Calif.; **David F. Mark**, Plainsboro, N.J.

[73] Assignee: **Hoffmann-LaRoche Inc.**, Nutley, N.J.

[21] Appl. No.: **413,623**

[22] Filed: **Sep. 28, 1989**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 396,986, Aug. 21, 1989, abandoned.

[51] **Int. Cl.**[5] **C12Q 1/68**; C12P 19/34; C12N 15/00; C07H 21/04

[52] **U.S. Cl.** **435/6**; 435/91; 536/24.33; 935/77; 935/78

[58] **Field of Search** 435/6, 810, 91; 536/27; 935/77, 78; 436/501, 808

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Smith et al., 1989,*J. Immunological Methods* 118:265–272.
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(List continued on next page.)

Primary Examiner—Margaret Moskowitz
Assistant Examiner—Mindy B. Fleisher
Attorney, Agent, or Firm—George M. Gould; Dennis P. Tramaloni; Stacey R. Sias

[57] **ABSTRACT**

The present invention provides a method for determining the amount of a target acid segment in a sample by polymerase chain reaction. The method involves the simultaneous amplification of the target nucleic acid segment and an internal standard nucleic acid segment. The amount of amplified DNA from each segment is determined and compared to standard curves to determine the amount of the target nucleic acid segment present in the sample prior to amplification. The method is especially preferred for determining the quantity of a specific mRNA species in a biological sample. Additionally, an internal standard is provided useful for quantitation of multiple mRNA species.

10 Claims, 4 Drawing Sheets

EXHIBIT N

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FIG. 1

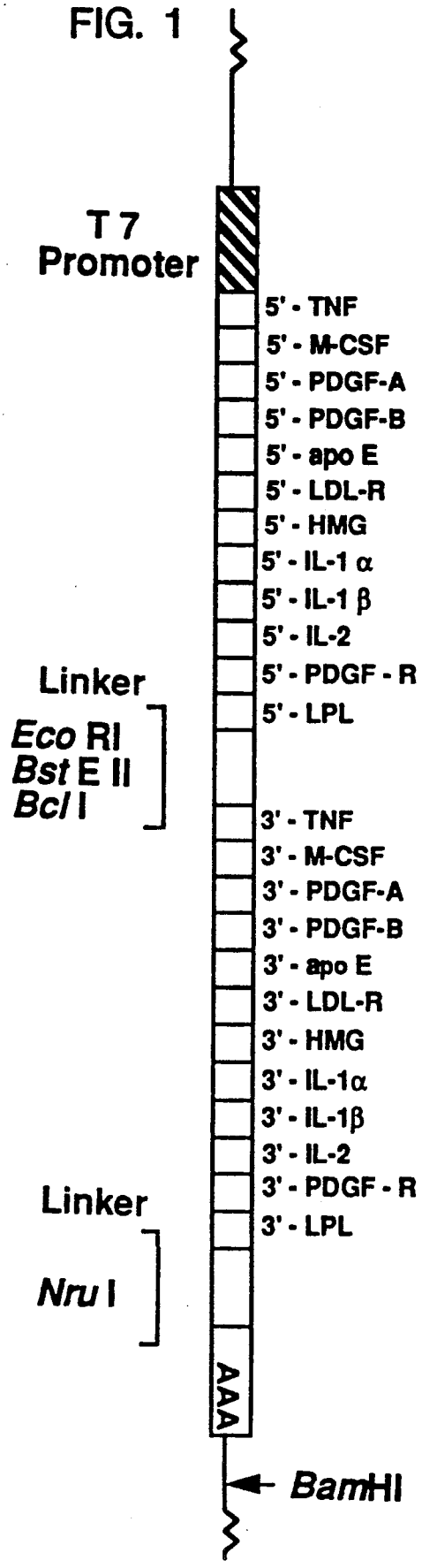

T 7
Promoter
5' - TNF
5' - M-CSF
5' - PDGF-A
5' - PDGF-B
5' - apo E
5' - LDL-R
5' - HMG
5' - IL-1 α
5' - IL-1 β
5' - IL-2
5' - PDGF - R
5' - LPL
Linker
Eco RI
Bst E II
Bcl I
3' - TNF
3' - M-CSF
3' - PDGF-A
3' - PDGF-B
3' - apo E
3' - LDL-R
3' - HMG
3' - IL-1α
3' - IL-1β
3' - IL-2
3' - PDGF - R
3' - LPL
Linker
Nru I
AAA
BamHI

FIG. 2A

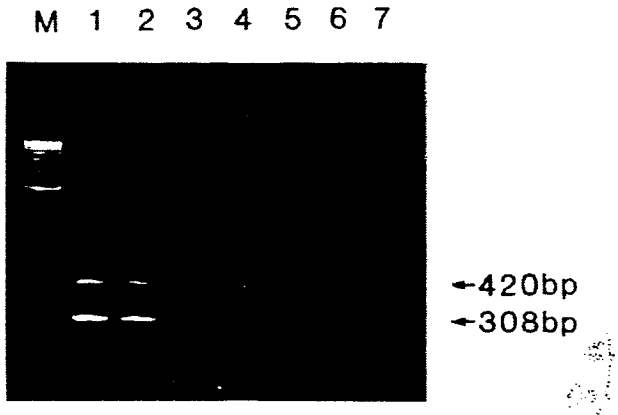


FIG. 2B

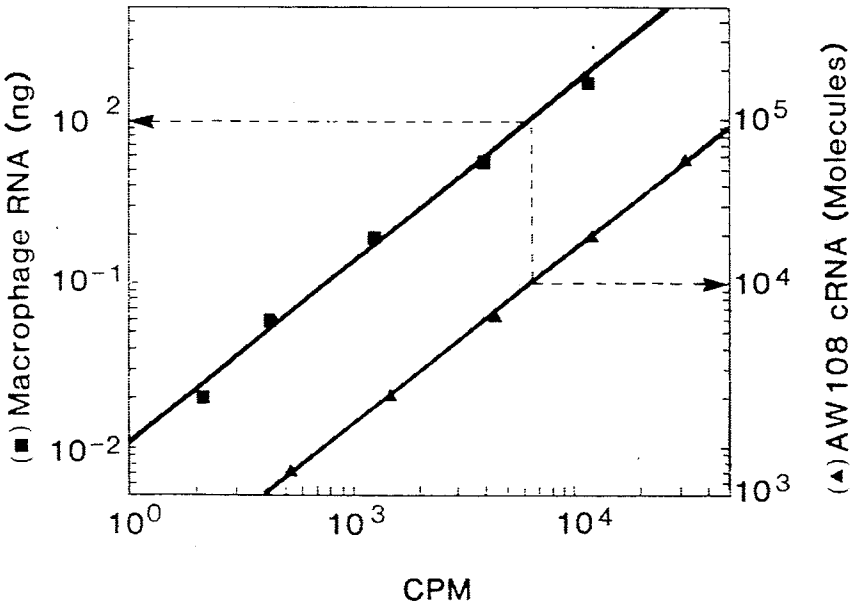


FIG. 2C

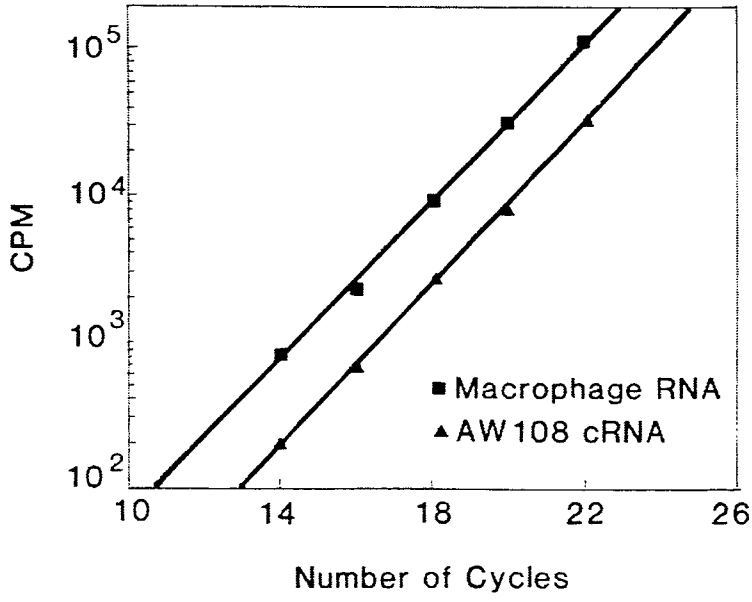
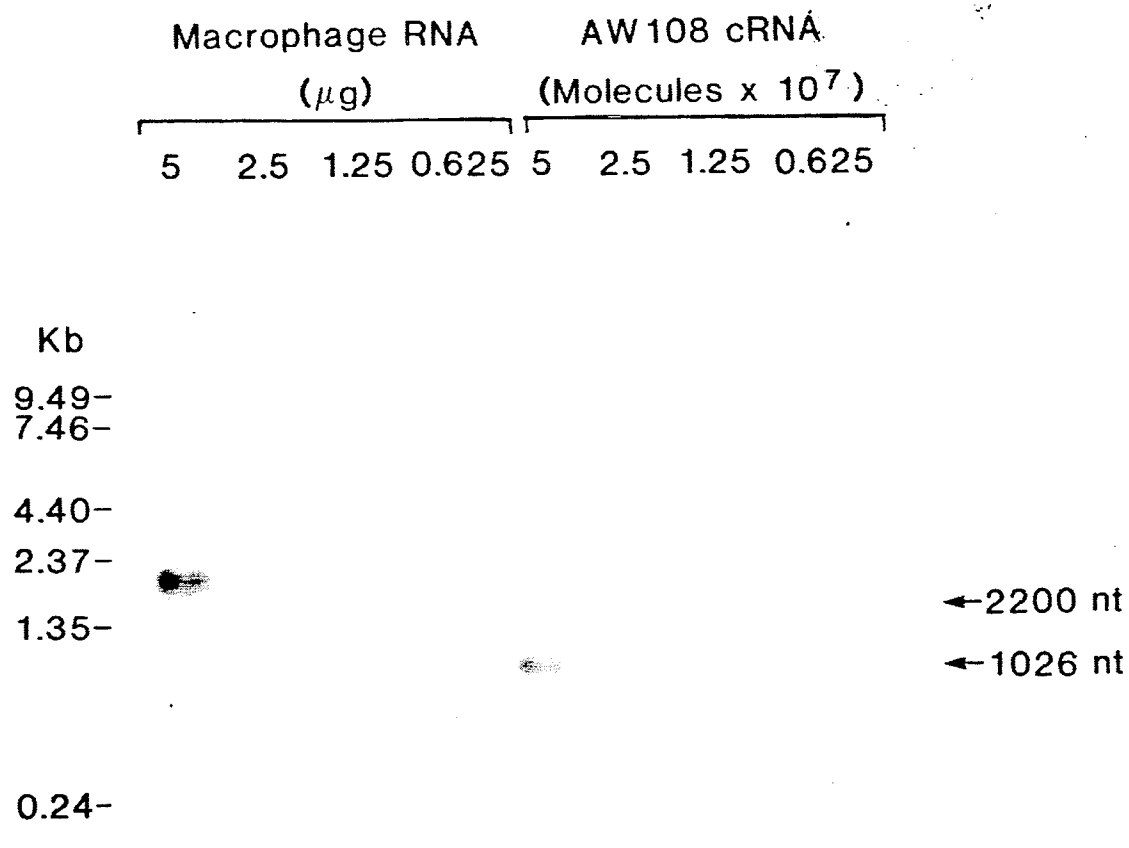


FIG. 3


Macrophage RNA
(μg)
5 2.5 1.25 0.625
AW108 cRNA
(Molecules x 10^7)
5 2.5 1.25 0.625
Kb
9.49-
7.46-
4.40-
2.37-
1.35-
0.24-
←2200 nt
←1026 nt

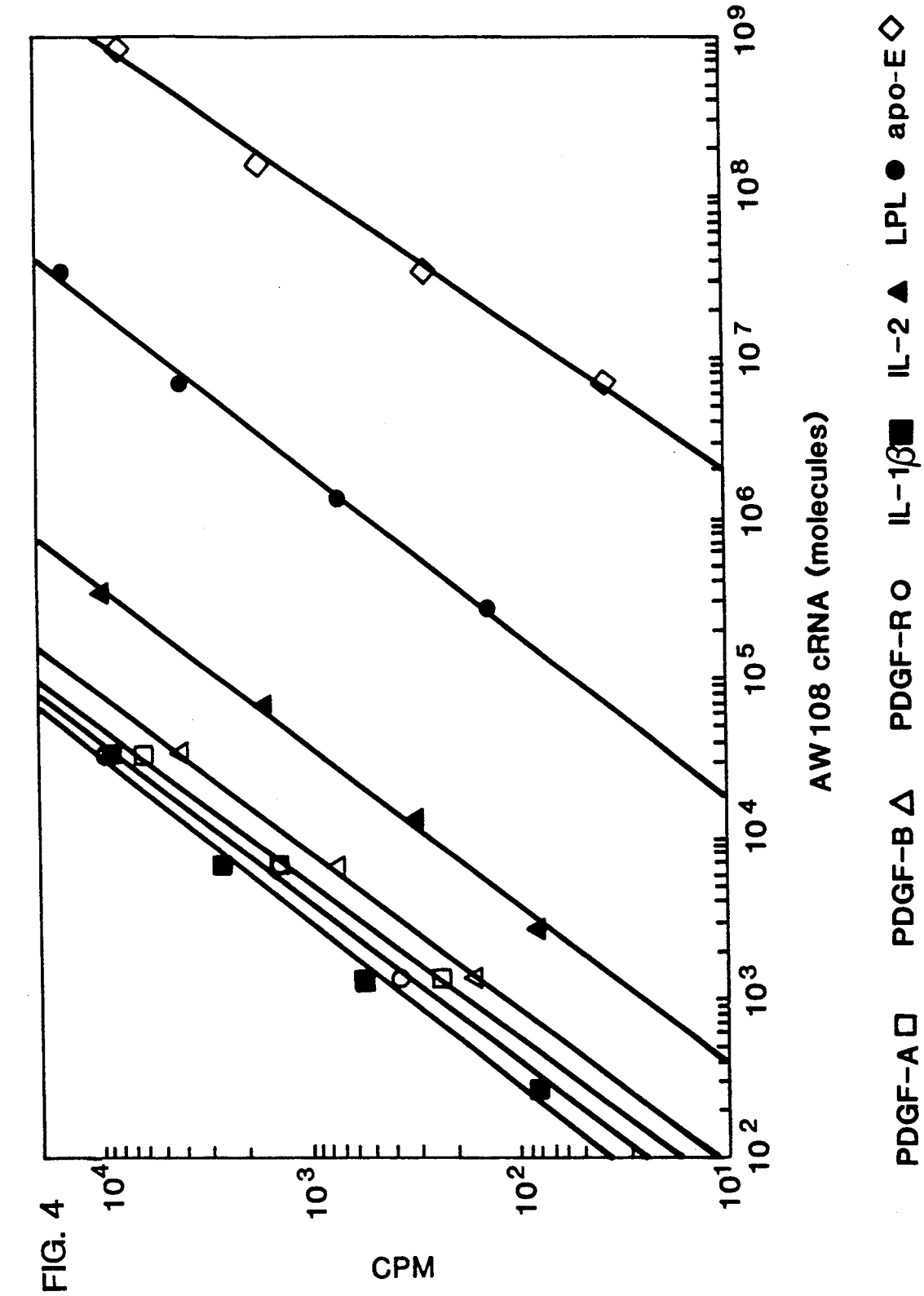

AW108 cRNA (molecules)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
CPM
10^4
10^3
10^2
PDGF-A □ PDGF-B △ PDGF-R ○ IL-1β ■ IL-2 ▲ LPL ● apo-E ◇

FIG. 4

QUANTITATION OF NUCLEIC ACIDS USING THE POLYMERASE CHAIN REACTION

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending U.S. Ser. No. 396,986, filed Aug. 21, 1989 now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the quantitative determination of a particular nucleic acid segment in a sample. The invention is particularly useful for determining the quantity of specific mRNA molecules in a biological sample. The method is therefore especially applicable in the field of medical diagnostics but can also be applied in the fields of genetics, molecular biology, and biochemistry.

2. Description of Related Disclosures

U.S. Pat. Nos. 4,683,195 and 4,683,202 disclose methods for carrying out the polymerase chain reaction (PCR), a nucleic acid amplification method, and for using PCR in the detection of specific nucleotide sequences. European Patent Office Publication (EPO) No. 258,017 describes Taq polymerase, a preferred DNA polymerase for use in PCR. These publications are incorporated by reference herein.

PCR methods have widespread applications in genetic disease diagnosis (see Wu et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:2757-2760 and Myerswitz, 1988, *Proc. Natl. Acad. Sci. USA* 85:3955-3959), as well as disease susceptibility and cancer diagnosis (see Horn et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:6012-6016; Todd et al., 1987, *Nature* 329:599-604; Kawasaki, 1988, *Proc. Natl. Acad. Sci. USA* 85:5698-5702; and Neri et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:9268-9272). However, these uses have provided only qualitative results by, for example, detecting unique mRNA transcripts from abnormal cells in a background of normal cells.

An attempt to use PCR for quantitative studies of mRNA level for thymidylate synthase in tumors has been published (see Kashani-Sabet, 1988, *Cancer Res.* 48:5775-5778). However, this study provides only relative comparisons of amounts of mRNA in biological samples. It has been much more difficult to quantitate the absolute amount of specific mRNA without an internal standard of known concentration. Other methods have been described for quantitating nucleic acid species by using PCR to co-amplify a second, unrelated, template cDNA (see Chelly et al., 1988, *Nature* 333:858-860 and Rappolee et al., 1988, *Science* 241:708-712). The use of an unrelated cDNA standard also necessitates the use of a second set of oligonucleotide primers, unrelated to the specific target mRNA.

Because amplification is an exponential process, small differences in any of the variables which control the reaction rate, including the length and nucleotide sequence of the primer pairs, can lead to dramatic differences in the yield of PCR product. Analyses which use two sets of unrelated primers, therefore, can only provide a relative comparison of two independent amplification reactions rather than an absolute measure of mRNA concentration.

Gilliland et al. (*J. Cellular Biochemistry*, UCLA Symposia on Molecular and Cellular Biology, Apr. 3-21, 1989, Abstract WH001) describe alternative approaches

to mRNA quantitation to avoid some of the problems associated with unrelated templates as amplification standards. However, the Gilliland et al. suggestions have other inherent limitations. One approach requires mapping of genomic introns and exons for the gene corresponding to a specific target mRNA. Gilliland et al. also proposes an alternative approach using site directed mutagenesis to construct an internal standard, which causes the formation of heteroduplexes following amplification. These heteroduplexes result in an over estimation of the amount of target sequence present in the original sample. Smith et al. (Smith et al., 1989, *J. Immunol. Meth.* 118:265-272) have used an RNA dot blot assay to asses quantitatively the expression level of the two IL-1 mRNAs in human macrophages. Smith et al. reported that the level of sensitivity for IL-1α mRNA was approximately 10^7 molecules by his method, and IL-1α mRNA was undetected in uninduced macrophages. The present invention provides a quantitation method which can readily measure 10^4 molecules and readily detects IL-1α mRNA in uninduced as well as induced macrophages in a sample assay. This 1000 fold increase in sensitivity represents an important advance in quantitative analysis for clinical and research purposes.

There remains a need for a method to quantitate directly, accurately, and reproducibly the amount of a specific nucleic acid segment in a sample. The availability of quantitative PCR will provide valuable information in a number of research areas. More particularly, the invention provides critical information in disease diagnosis and cancer therapy. For example, a reliable, sensitive, quantitative analysis can be critical in determining the extent of induction of mRNA synthesis in response to exogenous stimuli. The present invention overcomes the numerous limitations inherent in the attempts of others in this field, and thus provides means for accurately quantifying the amount of a nucleic acid segment in a biological sample.

SUMMARY OF THE INVENTION

The present invention provides a method for quantifying a target nucleic acid segment in a sample, which method comprises the steps of:

(a) adding to said sample an amount of standard nucleic acid segment;

(b) treating said sample under conditions suitable for carrying out a polymerase chain reaction, wherein said nucleic acids are rendered single-stranded and exposed to an agent for polymerization, deoxynucleoside 5' triphosphates, and a pair of oligonucleotide primers, wherein said pair of primers is specific for both the target and standard nucleic acid segments, such that an extension product of each primer of said pair can be synthesized using separate strands of the target and standard segments as a template for synthesis, such that the extension product of one primer, when it is separated from the remplate strand, can serve as a template for the synthesis of the extension product of the other primer of said pair;

(c) separating the primer extension product from the templates on which they were synthesized to form single-stranded molecules;

(d) repeating steps (b) and (c) on the single stranded molecules produced in step (c) at least once, whereby each repeat of steps (b) and (c) is one amplification cycle;

(e) measuring the amounts of the amplified target and standard segments produced in step (d); and

(f) calculating from the amplified target and standard segments produced in step (d) the amount of said target nucleic acid segment present in the sample before amplification.

The present invention also provides a plasmid useful for providing an internal standard for quantitation of target nucleic acid segments, said plasmid comprising a DNA sequence, said DNA sequence further comprising sequences which are identical to DNA sequences contained within said target nucleic acid segments.

The present invention also provides kits for the quantitation of specific nucleic acid segments in a biological sample.

DESCRIPTION OF THE FIGURES

FIG. **1** shows the positions of the 5′ primers and 3′ primers of Table I as they are arranged in plasmids AW108 and AW109. Other features are shown as they relate to the present invention.

In FIG. **2**A–C, the amount of IL-1α mRNA present in lipopolysaccharide (LPS) induced and uninduced macrophages was determined using the IL-1α primer pair.

FIG. **2**A depicts an ethidium bromide stained acrylamide gel wherein the amplified standard and target DNA segments are visible.

FIG. **2**B plots the amounts of standard and target IL-1α PCR product DNA produced against template concentrations.

FIG. **2**C shows a plot of the amounts of standard and template IL-1α PCR product DNA produced versus the number of amplification cycles.

FIG. **3** shows the results of a Northern blot containing samples of AW108 cRNA, and RNA isolated from LPS induced macrophages. The blot was probed with the IL-1α 3′ primer.

FIG. **4** shows the efficiency of amplification for different primer sets using the same cRNA template under the same conditions.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for determining the absolute amount of a nucleic acid segment in a sample. The method involves amplification, by a polymerase chain reaction, of two different segments of nucleic acid combined in one reaction mix. The two segments include a target segment and an internal standard segment. The internal standard is amplified using the same oligonucleotide primer pair as the target nucleic acid; however, the two nucleic acid segments yield amplified products which are distinguishable by size.

The standard segment is present in a known amount. Following amplification, the amount of each of the two polymerase chain reaction products is measured, and the amount of the target segment present in the original sample is quantitated by extrapolating against a standard curve. In addition, the internal standard described herein contains primer sequences for multiple genes, so that the same standard can be used to quantitate a number of different nucleic acid segments of interest.

The present invention has particular utility in providing a rapid, sensitive, and reliable method for accurately determining the quantity of low abundance, specific mRNAs present in a sample containing less than 0.1 ng of total RNA. The method provides an approach powerful enough to enable a measurement of heterogeneity of expression levels of specific mRNAs within particular subpopulations even at the single cell level.

By co-amplification of the target nucleic acid and the internal standard nucleic acid, variable effects are internally controlled and affect the yield of PCR product equally for target and standard nucleic acids. Numerous variables influence the rate of the PCR reaction. Such variables may include the concentrations of polymerase, dNTPs, $MgCl_2$, nucleic acid templates, and primers, as well as the rate of "primer-dimer" formation and tube-to-tube variations.

The amount of the target nucleic acid segment present in the sample prior to amplification is determined using a standard curve. The standard curve is generated by plotting the amount of the standard segment produced in a polymerase chain reaction against varying, but known, amounts of the RNA present before amplification. For accuracy, the amount of standard segment present before amplification is varied by serial dilution of the co-amplification reaction mix. The amount of target segment produced in the polymerase chain reaction is then compared to the standard curve to determine the amount of target segment present in the sample prior to amplification. Alternatively, the standard curve may be generated by plotting the amount of standard and target segments produced against the number of amplfication cycles. To ensure accuracy, it is preferred that the number of amplification cycles is varied by removing aliquots from one co-amplification reaction mixture after different numbers of amplification cycles have been completed.

The method of the invention is far superior to determinations of the amount of a nucleic acid segment in a sample as a relative, rather than absolute, amount. Further, the method is far more accurate than when an absolute amount is derived by employing a second set of oligonucleotide primers to amplify the standard, wherein that set of primers is different from the set used to amplify the target segment.

The method of the present invention is useful for quantifying a target RNA or DNA molecule. For determining an amount of DNA present in a sample, amplification methods described herein can be applied directly. As the examples disclosed below will demonstrate, the present invention is also useful in determining the amount of a specific mRNA in a sample of total RNA. The internal standard nucleic acid segment is provided on a DNA plasmid. The presence of an appropriately placed T7 polymerase promoter or another suitable promoter, such as the SP6 promoter, allows the plasmid to be used as a template for cRNA synthesis. As defined herein for the purpose of the present invention, the term "cRNA" refers to a ribonucleic acid segment synthesized from a DNA template by an RNA polymerase. Further, the plasmid may contain a polyadenylation sequence at the 3′ end to facilitate purification and subsequently quantitation of the in vitro synthesized cRNA. As described in the preferred embodiments, the DNA template is either plasmid AW108 or AW109, and the RNA polymerase is T7 polymerase. In one embodiment AW108 cRNA is synthesized as a sense strand from pAW108 by T7 polymerase. The structure of pAW108 is shown in FIG. **1**. The primer array as shown in FIG. **1** is identical for both pAW108 and pAW109. The cRNA molecule then serves as the internal standard template for reverse transcription by the

DNA polymerase, reverse transcriptase. Reverse transcriptase generates a cDNA transcript from an RNA template. The preferred embodiment of the invention, the internal standard cRNA, is synthesized as a sense strand. Following reverse transcription of the target mRNA and the standard cRNA, PCR is then performed.

As will be obvious to those skilled in the art, numerous methods are known for constructing plasmids useful in the method of the present invention. Higuchi, 1988, *Nucleic Acids Research* 16:7351-7367 and Ho, 1989, Gene 77:51-59 describe two methods for engineering novel plasmids which incorporate desired synthetic DNA sequences. Alternatively, synthetic DNA segments can be inserted via restriction enzyme digestion and ligation with an appropriately treated parent plasmid or phage vector. The internal standard of the preferred embodiment, pAW108, contains multiple primer sets which allow a single cRNA standard to be used to quantitate a number of different mRNAs. The presence of unique restriction enzyme sites in the pAW108 plasmid provides the flexibility to add new primer sets to the plasmid. The unique BamHI site is used to linearize the plasmid to produce a linear template for reverse transcription. A deposit of *E. coli* containing plasmid AW108 has been deposited with the American Type Culture Collection (ATCC) at 12301 Parklawn Drive, Rockville, Md. according to the terms of the Budapest Treaty. A deposit of *E. coli* containing plasmid AW109 has also been deposited with the American Type Culture Collection (ATCC) at 12301 Parklawn Drive, Rockville, Md. according to the terms of the Budapest Treaty.

Plasmid AW108 is derived from pcDV1 and pL1 which are disclosed in Okayama and Berg, 1983, *Mol. Cell Biol.* 3:280-289. The SV40 promotor region from pLI was inserted into pcDV1 as directed in the referenced article. The T7 promoter, synthetic oligonucleotide sequences, and a polyadenylation region from the IL-1α gene were then inserted to provide the AW108 plasmid as an internal standard for the quantitation of twelve specific mRNAs. The plasmid was transformed into *E. coli* and grown in Luria Broth with ampicillin at 50 μl/ml added.

Plasmid AW108 was subsequently used as the starting material to construct pAW109. A culture of *E. coli* containing pAW108 was grown, and plasmid DNA was purified by standard means. The plasmid was digested with BamHI and BglII restriction endonucleases, and the 1 kb fragment was purified. This fragment contained the 5′ and 3′ primer arrays shown in FIG. **1** as well as the polyadenylation sequence. Plasmid pSP72 (Promega Biotec, Madison, Wis. contains a T7 promoter adjacent to a polylinker to facilitate cloning. The plasmid also contains the ampicillin resistance gene.

The BglII-BamHI fragment from pAW108 was ligated into BglII and BamHI cleaved pSP72. Both of these are unique restriction sites within the polylinker region. The ligation mixture was used to transform *E. coli* DH5α, and resultant ampicillin resistant colonies were selected. The plasmid was assayed for the correct orientation of the BglII-BamHI insert. The resulting plasmid, pAW109, is suitable as an internal standard for mRNA quantitation.

As will be obvious to those skilled in the art, numerous other plasmids are available for insertion of desired DNA sequence to provide an internal standard useful in the present invention. Generally, the methods for transformation of such plasmids into a suitable host strain, propagation of the transformed host, and preparation of plasmid DNA as required for practice of the invention can be found in Maniatis et al., *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1985.

As used herein, the term "5′ primer" refers to an oligonucleotide comprising a sequence identical to the sequence contained within the sense strand of a target nucleotide segment. As used herein the term "3′ primer" refers to an oligonucleotide comprising a sequence complementary to a sequence contained within the sense strand of the same target nucleotide segment. Thus, a 3′ primer useful in the method of the present invention will hybridize to an mRNA, cRNA, or DNA template. It is further descriptive of the 3′ and 5′ primers that for both the internal standard cRNA and the target mRNA segment, the region of 3′ primer hybridization is located 3′ to the region of 5′ primer hybridization.

The 3′ and 5′ primers function in the method of the present invention as follows: the 3′ primer primes DNA synthesis in a PCR reaction to produce an antisense DNA strand, which provides a template for second strand DNA synthesis when the 5′ primer is included in the PCR reaction. Such a 5′ and 3′ primer are referred to herein as a "primer pair."

In the preferred embodiment, most members of a primer pair are designed to span two exon-intron junctions within the gene encoding each target mRNA. In this way the primers will only hybridize effectively to the desired target mRNA. Thus, small amounts of contaminating genomic DNA in a biological sample will not effect accurate quantitation of the target mRNA.

Thus, a primer pair will function in a PCR reaction to amplify a segment of nucleic acid having a primer sequence identical to a DNA segment contained within the standard nucleic acid, i.e., as illustrated here, plasmids AW108 and AW109. As described herein, both plasmids contain a DNA sequence which comprises the DNA sequence of twelve primer pairs arranged as follows: DNA identical in sequence to the 5′ primers of twelve target mRNAs is followed by the complementary DNA sequence of the 3′ primers for the same twelve target mRNAs (FIG. **1**). The primer pair DNA sequence within pAW108 and pAW109 corresponds to mRNAs encoding tumor necrosis factor (TNF), macrophage-colony stimulating factor (M-CSF), platelet-derived growth factor A (PDGF-A), platelet-derived growth factor B (PDGF-B), low density lipoprotein receptor (LDL-R), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-2 (IL-2), type beta platelet-derived growth factor receptor (PDGF-βR), and lipoprotein lipase (LPL). The primer pairs useful for amplifying the internal standard provided by AW108 or AW109 cRNA in the practice of the method of the invention are depicted in Table I.

TABLE I

Oligonucleotides of 12 Target Genes' 5' Primers and 3' Primers

mRNA Species	5' Primers	3' Primers	Size of PCR Product (bp) mRNA	cRNA
TNF	5'-CAGAGGGAAGACTTCCCCAG-3'	5'-CCTTGGTCTGGTAGGAGACG-3'	325	301
M-CSF	5'-GAACAGTTGAAAGATCCAGTG-3'	5'-TCGGACGCAGGCCTTGTCATG-3'	171	302
PDGF-A	5'-CCTGCCCATTCGGAGGAAGAG-3'	5'-TTGGCCACCTTGACGCTGCG-3'	225	301
PDGF-B	5'-GAAGGAGCCTGGGTTCCCTG-3'	5'-TTTCTCACCTGGACAGGTGG-3'	217	300
apo-E	5'-TTCCTGGCAGGATGCCAGGC-3'	5'-GGTCAGTTGTTCCTCCAGTTC-3'	270	301
LDL-R	5'-CAATGTCTCACCAAGCTCTG-3'	5'-TCTGTCTCGAGGGGTAGCTG-3'	258	301
HMG	5'-TACCATGTCAGGGGTACGTC-3'	5'-CAAGCCTAGAGACATAATCATC-3'	246	303
IL-1α	5'-GTCTCTGAATCAGAAATCCTTCTATC-3'	5'-CATGTCAAATTTCACTGCTTCATCC-3'	420	308
IL-1β	5'-AAACAGATGAAGTGCTCCTTCCAGG-3'	5'-TGTTTCAGATCCCTTTAGTTCCAG-3'	388	306
IL-2	5'-GAATGGAATTAATAATTACAAGAATCCC-3'	5'-TCCAGAACACCACTTGTTGCTCCA-3'	222	305
PDGF-R	5'-TGACCACCCAGCCATCCTTC-3'	5'-GAGGAGGTGTTGACTTCATTC-3'	228	300
LPL	5'-GAGATTTCTCTGTATGGCACC-3'	5'-CTGCAAATGACACTTTCTC-3'	277	300

TNF, tumor necrosis factor; M-CSF, macrophage-colony stimulating factor; PDGF-A, platelet-derived growth factor A; PDGF-B, platelet-derived growth factor B; apo-E, apolipoprotein E; LDL-R, low density lipoprotein receptor; HMG, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-2, interleukin-2; PDGF-R, type β platelet-derived growth factor receptor; LPL, lipoprotein lipase.

Other mRNA targets which may be readily quantitated in biological samples by the present invention include, but are not limited to, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), acidic-fibroblast growth factor (aFGF), basic-fibroblast growth factor (bFGF), c-McDonough feline sarcoma (c-fms), transforming growth factor-β (TGF-β), leukocyte adhesion protein-1 (LFA-1), interleukin-2 receptor-α (IL-2Rα), alpha-actin, desmin, β-actin, interleukin-6 (IL-6), interferon-α (IFN-α), interferon-γ (IFN-γ), interleukin-6 receptor (IL-6R), platelet derived growth factor-α receptor (PDGF-αR), interleukin-2 receptor-β (IL-2Rβ), interleukin-3 (IL-3), and interleukin-4 (IL-4) as well as human immunodeficiency virus (HIV). Examples of primer pairs useful for the detection and measurement of expression of these RNAs are exemplified by the oligonucleotide sequences shown in Table II.

TABLE II

G-CSF	5'GGTGAGTGAGTGTGCCACCT3', 5'GTTCTTCCATCTGCTGCCAG3';
GM-CSF	5'CACTGCTGCTGAGATGAATGAAACAG3', 5'GCACAGGAAGTTTCCGGGGTTGG3';
aFGF	5'TCCTTCCGGATGGCACAGTC3', 5'CATTTGGTGTCTGTGAGCCG3';
bFGF	5'GACCCTCACATCAAGCTACAAC3', 5'GGAAGAAAAGTATAGCTTTCTGC3';
c-fms	5'CAAGTATAAGCAGAAGCCCAAGTAC3', 5'GAGGGTCTTACCAAACTGCAGG3';
TGF-β	5'CATCAACGGGTTCACTACCG3', 5'TCCGTGGAGCTGAAGCAATAG3';
LFA-1	5'GAGTGCCTGAAGTTCGAAAAGG3', 5'CACACACTCTCGGCTCTCATC3';
IL-2Rα	5'GCTGCCAGGCAGAGCTCTGTGACG3', 5'GTTCCGAGTGGCAGAGCTTGTGC3';
α-actin	5'GCACAACTGGCATCGTGCTG3', 5'AGACTCCATCCCGATGAAGG3';
desmin	5'AGGAGAGCCGGATCAACCTTC3', 5'TCGCTGACGACCTCTCCATC3';
β-actin	5'CCTTCCTGGGCATGGAGTCCTG3', 5'GGAGCAATGATCTTGATCTTC3';
IL-6	5'CCTTCTCCACAAGCGCCTTC3', 5'GGCAAGTCTCCTCATTGAATC3';
IFN-α	5'AGCTGCAAGTCAAGCTGCTC3', 5'TCCCAAGCAGCAGATGAGTC3';
IFN-γ	5'GAAGAATTGGAAAGAGGAGAGTGACAGAAA3', 5'CATTCAAGTCAGTTACCGAATAATTAGTCAG3';
IL-6R	5'CATTGCCATTGTTCTGAGGTTC3', 5'AGTAGTCTGTATTGCTGATGTC3';
PDGF-αR	5'CTGGATGAGCAGAGACTGAG3', 5'AGGAAGCTGTCTTCCACCAG3';
IL-2Rβ	5'TTTCAGGTGCGGGTCAAGCCTCTG3', 5'AGTAACCCTGGTTGGTGAAGCAGC3';
IL-3	5'CATGAGCCGCCTGCCCGTCC3', 5'GGTTATTTTCCATCAGAATG3';
IL-4	5'CTCACCTCCCAACTGCTTCCC3', 5'GTGGAACTGCTGTGCAGTCGC3'; and
HIV	5'AGTGGGGGGACATC3', 5'TTTGGTCCTTGTCTTATG3'.

The PCR product from each primer set within pAW108 and pAW109 is 300–308 base pairs (bp), depending on the particular primer pair used. The 300–308 bp segment length of the illustrated example does not impose a limitation to the design of any internal standard. It is only necessary that the standard segment length is designed to be different in size from the PCR products of the target mRNAs and that the segment lengths be within the detection limits inherent in the

analytical system preferred (for example, acrylamide gel electrophoresis, agarose gel electrophoreses, or other chromatographic means). The size difference between the PCR amplification products permits easy separation of the internal standard cRNA amplification product from the target mRNA amplification product by, for example, gel electrophoresis. The unique BamHI site is used to linearize the AW108 or AW109 plasmid to produce cRNA transcripts. Such transcripts are useful for quantitation of a number of different specific mRNAs in, for example, treated and untreated samples. This method can be used to provide a transcriptional phenotype of a treated or untreated cell or tissue and thus provides for numerous clinical and research applications.

The cRNA and the target mRNA are reverse transcribed in the same reaction. In this way, the cRNA serves not only as a standard for mRNA quantitation, but also provides an internal mRNA control for the reverse transcription reaction. Reverse transcriptase requires a primer to initiate cDNA synthesis using an RNA template. In the practice of the present invention, this will be an oligonucleotide primer which hybridizes to both the standard cRNA and the target mRNA. The primer may be identical in sequence to the 3′ primer used for PCR amplification of that target mRNA. Alternatively, the primer for the reverse transcription reaction may be an oligonucleotide which hybridizes to the mRNA and cRNA at a position distal to the sequence of the 3′ amplification primer, for example, oligo (dT). Thus, the resultant cDNA contains within it a sequence identical to the sequence of the 3′ amplification primer. In the preferred embodiment disclosed herein, AW108 cRNA, as well as AW109 cRNA, contain a polyadenylation sequence at the 3′ end, and oligo (dT) is used as a primer for reverse transcription of the cRNA and mRNA templates. Additionally, oligo (dT) permits amplification of more than one target sequence from the same reverse transcriptase reaction mix.

The same primers are used in the PCR amplification of both the standard and target templates; therefore, there are no primer efficiency differences between amplification of the standard and the target RNAs. When dilution series of mixtures of the target mRNA and internal standard cRNA are amplified in the same tube, and the reaction is terminated in the exponential phase of the amplification, the amount of target mRNA that was present in the sample prior to amplification can be determined by extrapolating against the internal standard cRNA standard curve. The amount of DNA produced is plotted against the amount of starting material for both the standard and the target. The standard curve allows extrapolation of the target data to determine the amount of target in the starting material. This value may be expressed as molecules of target mRNA/ng total RNA. Alternatively, it may be determined as of percentage or an amount by weight, or as a copy number.

Alternatively, a method is provided for determining the amount of target mRNA by varying the number of amplification cycles. The amount of amplified products produced is plotted against the number of amplification cycles for both the standard and target segments. The plotted data illustrates that portion of the reactions wherein the rate of amplification is exponential. Therefore, a ratio of products formed can be equated to a ratio of starting materials to determine the initial amount of target segment present. This is done according to the formula:

$$\frac{N_{o(mRNA)}}{N_{o(cRNA)}} = \frac{N_{(mRNA)}}{N_{(cRNA)}}$$

where N_o is the initial amount of material, and N is the amount of amplified product produced.

In another embodiment of the present invention, a third primer array is inserted into the internal standard plasmid between the 5′ primer array and the 3′ primer array. The third oligonucleotide array is comprised of a series of synthetic sequences wherein there is one sequence corresponding to each RNA for which the plasmid contains a 5′ and 3′ primer pair. This array is designed such that for each target RNA to be quantitated, the amplified product will contain within it a sequence identical to a portion of the third oligonucleotide array. Thus, both the amplified target and amplified standard DNA segments contain an identical internal segment providing a probe hybridization site, whereby for each primer pair, an oligonucleotide probe is useful to detect the amplified target as well as the amplified standard DNA.

Where a third oligonucleotide array is included in the standard plasmid, the PCR reaction can be carried out without the use of label. It is preferred that the reverse transcription and amplification reactions are carried out in separate tubes for each of the standard and target templates, rather than as a co-amplification. Following amplification, amount of product is quantiated by use of a dot blot format employing a single-stranded oligonucleotide probe which has a sequence corresponding to the internal sequence provided by the third primer array.

As an illustrative example of the present invention, the AW108 internal standard was used to determine the amount of several lymphokine mRNAs, including IL-1α mRNA, isolated from lipoplysaccharide (LPS)-induced and control cultures of human macrophages. Lymphokine mRNA levels were also measured in human atherosclerotic plaque tissue.

As provided by the present invention, target mRNA is quantified most accurately by using an internal standard having, in part, the same sequence as the target itself. Quantification of mRNA sequences by PCR amplification using an unrelated template as an internal standard provides only comparative data because of differences in efficiency between the primer pairs for the standard and the target mRNAs. This is inherent in the amplification process because PCR amplification is an exponential process. The extent of amplification (N) is given by the equation: $N=N_o(1+\text{eff})^n$ where N_o is the initial amount of material, eff is the efficiency, and n is the cycle number. Thus, small differences in efficiency lead to large differences in the yield of PCR product and result in a misrepresentation of the amount of template present in a biological sample. Further, differences in primer efficiency are difficult parameters to regulate for quantitative analyses. The present invention overcomes these problems.

The significant contribution of primer efficiency in the accurate quantitation of a nucleic acid segment is underscored in an example below. AW108 cRNA was used as the template for PCR amplification of several different primer sets. The efficiency of amplification by these different primer sets, under the same PCR conditions, varies over a range of several orders of magnitude. This invention addresses itself to this issue, which

is clearly critical in any attempt to quantitate mRNA expression by PCR, and overcomes the problem of primer efficiency by using the same primers for amplification of the target mRNA and the internal standard cRNA.

The present invention requires that the amplification of the standard and target segments of nucleic acid be carried out in the same reaction. In the preferred embodiment of the present invention, the reverse transcriptase reaction of the standard cRNA and target mRNA is also carried out in the same reaction. Those skilled in the art will recognize from the foregoing that one could quantitate a target nucleic acid by performing the standard and target reverse transcriptase and amplification reactions separately. However, the accuracy of such a method is dependent on the degree to which the reverse transcription and amplification steps proceed with similar efficiency for both amplifications. By performing both reverse transcriptase reactions in the same tube and both amplification reactions in the same reaction tube, one ensures excellent accuracy.

The amount of an amplified DNA fragment in a given sample can influence amplification efficiency. When a high template concentration is used, or occurs as a result of the PCR amplification, phenomena can occur which are limiting factors for efficient amplification. Such phenomena include substrate saturation of enzyme, product inhibition of enzyme, incomplete product strand separation, and product strand reannealing. These problems are readily avoided, however, by an initial titration of the specific target mRNAs to find the range of concentrations that gives exponential amplification over a defined range of cycle numbers. Accordingly, to obtain reliable quantitative evaluation of specific mRNA using the described invention, the range of concentrations for both the standard and target templates, as well as the number of amplification cycles, should be such that the reactions remain within the exponential phase.

Thus, in the preferred embodiment, the reaction conditions described make use of 50 ng–1 μg of total cellular RNA combined with approximately 2×10^2–2×10^7 molecules of cRNA. As little as 50 pg cellular RNA is also suitable for purposes of the present invention. In the example described, as few as 1×10^4 molecules of IL-1α are detected. It is not necessary that mRNA be purified from a total RNA preparation in order to employ the method of the invention.

Samples suitable for analysis by this method may be of human or non-human origin; they may be derived from cultured samples, or isolated from dissected tissue or from cells of immunologically defined phenotype. The latter can be obtained by monoclonal antibody staining and fluorescence-activated cell sorter (FACS) isolation of enzyme-dissociated cells or by removal of specific areas from immunohistochemically stained slides. This will permit definitive identification of the cell types producing specific mRNAs.

The amount of amplified DNA generated in the method of the present invention can be measured in different ways. For instance, labeled primers wherein one or both members of any primer pair is labeled, or labeled nucleotides, can be used in PCR, and the incorporation of the label can be measured to determine the amount of amplified DNA. The label can be isotopic or non-isotopic. Alternatively the amount of amplified product can be determined by electrophoresis and visualization of the amplified product by staining or by hybridization with a labeled probe. Densitometry can be used to calculate the amount of product on a stained gel, or by extrapolation from an autoradiograph when labeled probe is used. When a labeled probe is used, the probe should be present in excess of the amplified product. In one such embodiment of the invention, primers are isotopically labeled and the resultant amplified products are electrophoresed on an acrylamide gel. The region where the product is expected to migrate is excised, and the amount of label present is determined by Cerenkov counting. The amount of label present is plotted versus the amount of known starting material.

The method of the invention requires that the amplified amounts of a template and standard segment produced in a single polymerase chain reaction be determined. Thus, the method requires that the amplified template segment be distinguishable from the amplified standard segment. If the segments are of different sizes, then it is relatively simple to distinguish one amplified segment from the other, i.e., the amplified products can be readily separated by gel electrophoresis. The present invention does not require that the amplified product be of different sizes, however, for other methods can be utilized to distinguish one amplified segment from another. For instance, the internal probe specific for one segment can be labeled differently than the internal probe specific for the other segment.

The quantitative method described herein is useful for analyses of in vivo biological samples. As is illustrated in the following example, quantitative PCR analysis of PDGF-A and B chain mRNA a human atherosclerotic lesion versus a normal blood vessel emphasizes the sensitivity of this approach in investigating the biology of cells and tissues in vivo. For example, when the present method was used to measure IL-1α and IL-1β mRNAs in atherosclerotic tissue, the results suggested that there may be inflammatory or immunological components in the pathogenesis of the disease.

Due to its high sensitivity, speed, and accuracy, the present quantitative PCR method can be used to study gene expression in a more extensive way than has been possible to date, allowing quantitative measurements of gene expression in a very small number of cells and from small amounts of tissue samples available from in vivo sources, such as biopsies. This technique can also provide information on changes in expression level of specific RNA molecules which may be valuable in the diagnosis and analysis of, for example, infectious disease states, cancer, metabolic disorders, and autoimmune diseases.

It will be apparent to those skilled in the art that the method of the present invention is amenable to commercialization as a kit for the quantitation of one or more nucleic acids in a sample. For example, in its simplest embodiment, such a kit would provide an internal standard and an appropriate oligonucleotide primer pair. In another embodiment, a kit may contain an internal standard, an appropriate oligonucleotide primer pairs, a DNA polymerase, a RNA polymerase, a reverse transcriptase, nucleotide triphosphates, restriction enzymes, buffers for carrying out cRNA and cDNA synthesis, restriction enzyme digests, and amplification by PCR. Further, the kits may contain a thermostable DNA polymerase; for example, the thermostable DNA polymerase Taq isolated from *Thermus aquaticus* as an agent of polymerization.

The method of the invention is exemplified below, but those skilled in the art will recognize the present

invention is broadly applicable and in no way limited to the specific embodiments described below.

EXAMPLE 1

Methods

A. Preparation of Internal Standard and RNAs

A synthetic gene was constructed using a technique of oligonucleotide overlap extension and amplification by PCR. The procedure used was similar to that described by Ho et al. for use in site-directed mutagenesis (Ho et al., 1989, *Gene* 77: 51–59). After construction, the synthetic gene was subcloned into an Okayama-Berg vector containing the T7 polymerase promoter and a polyadenylated sequence. The resulting plasmid, AW108, is shown in FIG. **1**. This plasmid was used as a template for transcription by T7 polymerase according to the transcription protocol of the manufacturer (Promega Biotec, Madison, Wis. The resulting AW108 cRNA product was purified by oligo(dT) chromatography and gel electrophoresis. Alternatively, pAW109 was used to prepare a cRNA standard. The cRNA product was purified by selective elution using the QIAGEN-tip spin column hybridization separator system (QIAGEN Inc., Studio City, Calif. followed by oligo(dT) chromatography. The Qiagen-tip was used according to manufacturer's instructions for purification of RNA and run off RNA transcripts.

For either AW108 cRNA or AW109 cRNA, the purified cRNA was quantitated by determining absorbance at 260 nm. The number of molecules present was determined based on the molecular weight of the transcript. AW108 cRNA is 1026 nucleotides in length, therefore, 1 mole = 3.386×10^5 gm (1026×330). Thus, 3.386×10^5 gm contains 6×10^{23} cRNA molecules. The number of molecules in 1 pg of AW108 cRNA is $(6\times10^{23})/(3.386\times10^5\ \text{gm})=1.77\times10^6$.

Total cellular RNA was isolated from macrophages and tissues by the method of acid guanidium thiocyanate-phenol-chloroform extraction according to Chomczynski et al., 1987, *Analyt. Biochem.* 162:156–159.

B. Purification of cRNA by Gel Electrophoresis

The cRNA prepared from pAW108 was electrophoresed in 1% low melt agarose, ultra pure grade, in TBE buffer. The region of the gel corresponding to 1 kb was cut out of the gel and melted in 0.2–0.4 ml of 0.1M NETS buffer (0.1M NaCl, 0.01M EDTA; 0.01M Tris-HCl, pH 7.4; 0.2% SDS) containing 1 mM 2-ME, in a water bath at 95° C. for 3–5 minutes and solidified quickly in an ice bucket. The samples were then frozen at −70° C. for at least two hours.

The frozen samples of melted agarose were thawed at 37° C. and centrifuged at top speed in an eppendorf centrifuge kept in the cold room. The agarose was pelleted out. The supernatant liquid was transferred to another eppendorf tube and extracted with a mixture of 100 μl phenol chloroform containing 1% isoamhl alcohol. The phenol was saturated with 0.1M NETS buffer. The aqueous phase was collected, and the RNA was ethanol precipitated. The RNA pellet was washed with 0.1 ml of 2M LiCl and then with 0.1 ml ethanol. The RNA was dried and then dissolved in an appropriate amount of sterile distilled water (2–100 μl) and was ready for reverse transcription.

C. Oligonucleotides Used for Amplification

Oligonucleotides were synthesized on a Biosearch (San Rafael, CA) DNA synthesizer. Most of the primers are RNA-specific primers. The 5′ primers spanned the junction of the first two exons and the 3′ primers spanned the junction of the next two exons. Alternatively, the 5′ primers spanned the junction of the first and second exons and the 3′ primers spanned the junction of the second and third exons. These sequences, the genes to which they correspond, and the sizes of amplified products obtained using the primers are shown in Table I.

D.cDNA Preparation

RNA was reverse transcribed into cDNA as previously described (see Gerard, 1987, *Focus* (Bethesda Research Labs) 9:5). A 10 μl reverse transcription reaction, containing 1 μg of total cellular RNA, $1.77\times10^2-1.77\times10^6$ molecules of AW108 cRNA, 1×PCR buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 μg/ml BSA), 1 mM DTT, 0.5 mM dNTP, 10 units RNasin (Promega Biotec), 0.1 μg oligo $(dT)_{12\text{-}18}$, and 100 units of BRL Moloney MuL V reverse transcriptase (Bethesda Research Laboratories) was prepared. The reaction was incubated at 37° C. for 60 minutes, heated to 95° C. for 5–10 minutes, then quickly chilled on ice.

E. Amplification Procedure

One tenth of the cDNA reaction mixture was diluted in a three-fold dilution series with 0.1 μg/μl tRNA, followed by adjustment to a final concentration of 1×PCR buffer, 50 μM dNTPs, 0.1 μM each of 5′ and 3′ primers, 1×10^6 cpm of ^{32}P end-labeled primer and 1 unit of Taq DNA polymerase (Perkin-Elmer Cetus) in a total volume of 50 μl. The mixture was overlaid with 100 μl mineral oil to prevent evaporation and then amplified for 25 cycles with the Perkin-Elmer Cetus Thermal Cycler. Alternatively, one tenth of the cDNA reaction mixture was amplified using the same conditions as above with varying numbers of cycles. The amplification profile involved denaturation at 95° C. for 30 seconds, primer annealing at 55° C. for 30 seconds, and extension at 72° C. for 1 minute. Oligonucleotides were labeled with γ-^{32}P-ATP by using polynucleotide kinase and unicorporated nucleotides were removed on a Bio-Gel P-4 column.

F. Quantitative Analysis

Ten μl of each PCR reaction mixture were electrophoresed in 8% polyacrylamide gels in Tris/-borate/EDTA buffer. Gels were stained with ethidium bromide and photographed under UV-light irradiation. Appropriate bands were cut from the gel, and radioactivity was determined by Cerenkov counting. The amount of radioactivity recovered from the excised gel bands was plotted against the template concentrations. Data were plotted by exponential curve fitting with a Slide-Write Plus program (Advanced Graphics Software) The amount of target mRNA was quantitated by extrapolating against the AW108 cRNA internal standard curve.

G. Northern Blot Analysis

RNA was electrophoresed in a 1.5% agarose gel containing formaldehyde and transferred to a nitrocellulose filter in 20×SSC (1×SCC contained 0.15M sodium chloride and 0.015M sodium citrate). The blot was hybridized with 2×10^6 cpm of ^{32}P end-labeled oligonucleotides per ml. Hybridization was for 4 hours at 55° C.

in 0.75M NaCl, 0.075M sodium citrate, pH 7.0, 20 mM sodium phosphate, pH 7.0, 5 mM EDTA, 200 μg yeast RNA per ml, and 1% sarkosyl (Sigma). The blot was washed in 1×SSC at 55° C. for 30 minutes and autoradiographed with intensifying screens at −70° C.

H. Macrophage Cultures

Human peripheral blood monocytes were isolated from buffy coat preparations by Ficoll/Hypaque gradient centrifugation followed by adherence to plastic for one hour. Adherent cells were then removed and replated at 10^6 cells/well onto 6 well plates in RPMI 1640 medium supplemented with 2% fetal calf serum and 2000 units/ml recombinant macrophage-colony stimulating factor (Cetus Corporation). After ten days, half of the cultures were treated with 5 μg/ml LPS (Sigma). All the cultures were harvested for nucleic acid isolation 5 hours later.

I. Human Tissue Samples

The carotid endarterectomy sample was obtained during the course of a surgical operation with the informed consent of the patient. The RNA preparation of a histologically normal coronary artery was recovered from a heart transplant recipient.

EXAMPLE 2

Quantification of IL-1α in a Preparation of Human Macrophage Total RNA

As an example of the present method, the AW108 internal standard was used to determine the amount of IL-1α mRNA isolated from LPS-induced cultures of human macrophages. Two different protocols were used to conduct this analysis. In the first case, the amount of template and standard RNAs was varied by serial dilution to generate a standard curve. In the second case, the number of amplification cycles was varied and plotted against the amount of PCR product.

A. Quantification of mRNA By Varying The Amount Of Internal Standard

Fifty ng of total macrophage RNA and 1.77×10^6 molecules of AW108 cRNA were combined and then reverse transcribed into cDNA. Serial three-fold dilutions of one tenth of the cDNA mixture were amplified using the IL-1α specific primers listed in Table 1. About 1×10^6 cpm of ^{32}P end-labeled 5′ primer were included in the amplification. Reaction products were resolved by gel electrophoresis and visualized by ethidium bromide staining (FIG. 2A). The amounts of radioactivity recovered from the excised gel bands were plotted against the template concentrations (FIG. 2B). In this experiment, target mRNA and AW108 cRNA were amplified after serial three-fold dilutions, and the results demonstrate that the method can resolve less than three-fold differences in RNA concentrations. The fact that the reaction rates of AW108 cRNA and IL-1α mRNA amplification are identical within this exponential phase of the PCR reaction allows construction of a standard curve for AW108 cRNA and extrapolation to a copy number for the IL-1α mRNA present in the macrophages. As shown in FIG. 2B, 1 ng of LPS-induced macrophage total RNA and 1×10^4 molecules of AW108 cRNA gave the same amount of IL-1α PCR product. In other words, 1 ng of LPS-induced macrophage RNA contained 1×10^4 molecules of IL-1α mRNA.

B. Quantification of mRNA by Varying The Number of Amplification Cycles

Five hundred ng of total macrophage RNA were reverse transcribed with 1.77×10^6 molecules of AW108 cRNA. Aliquots containing one tenth of the cDNA mixture each were subjected to 14, 16, 18, 20, 22, 24, 26, or 28 cycles of amplification under the same conditions as in protocol E. The amounts of radioactivity recovered from the excised bands were plotted as a function of the number of cycles (FIG. 2C). The rates of amplification were exponential between 14 and 22 cycles for both templates. At 24, 26, and 28 cycles, the rates decreased drastically and approached a plateau. The efficiency of amplification was calculated from the slopes of these curves and found to be 88% for both AW108 cRNA and IL-1α mRNA. Because the amplification efficiency was the same for both co-amplified targets within the exponential phase, the absolute value of IL-1α mRNA can be calculated by comparison with the AW108 cRNA internal standard employing the formula:

$$\frac{N_{o(mRNA)}}{N_{o(cRNA)}} = \frac{N_{(mRNA)}}{N_{(cRNA)}}$$

where N_o is the initial amount of material, and N is the extent of amplification. The amount of IL-1α mRNA in 1 ng of LPS-induced macrophage total RNA calculated by this method was 1.1×10^4 molecules. Thus, the results using either of these two alternative protocols for quantitation are the same.

C. Correlation of PCR Results with Northern Analysis

The amount of IL-1α mRNA in LPS-induced macrophages determined by the quantitative PCR method was verified by Northern blot analysis. The PCR analysis (see above) demonstrated that 1 ng of macrophage RNA and 1×10^4 molecules of AW108 cRNA produced the same amount IL-1α PCR product. Thus, 5 μg of macrophage RNA and 5×10^7 molecules of AW108 cRNA should give similar signal intensities by Northern analysis. Two-fold serial dilutions of macrophage RNA and AW108 cRNA were subjected to Northern blot analysis by probing with the IL-1α 3′ primer. The sizes of the target RNA molecules were estimated to be ~2,200 nucleotides for IL-1α mRNA in macrophages and 1026 nucleotides for AW108 cRNA. Hybridization signals of equal intensity were detected at all the dilutions of macrophage RNA and AW108 cRNA, as shown in FIG. 3. This result demonstrates that the amount of mRNA estimated by the quantitative PCR method correlates with the results of Northern analysis.

EXAMPLE 3

Effect of Primer Efficiency Differences

There are many variables which could influence the effciency of the PCR amplification. Some of the parameters which can be controlled easily are the concentrations of template, dNTPs, $MgCl_2$, primers, polymerase, and PCR cycle profile. However, differences in primer efficiency are difficult parameters to regulate for quantitative analyses. To analyze the primer efficiency effect in the quantitative PCR method, AW108 cRNA was used as the template for PCR amplification of seven different primer sets: IL-1β, PDGF-B, PDGF-B, PDGF-R, IL-2, LPL, and apo-E. As indicated in FIG.

4, the efficiency of amplification by these different primer sets under the same PCR amplification conditions varied over a range of several orders of magnitude. For instance, the IL-1β primers are 10^5-fold more efficient than the apo-E primers. Thus, it is critical to use the same primers for amplification of the target mRNA and the internal standard in any attempts to quantitate mRNA expression by PCR.

EXAMPLE 4

Quantitation of Specific mRNAs in Untreated and LPS-Induced Macrophages

A major advantage of the present PCR quantitative techniques is that the method enables one to analyze several target mRNA species in parallel. Table III shows the results from quantitation of the expression levels of six cytokine mRNAs in human macrophages in response to LPS treatment. The levels of IL-1β and IL-1α mRNAs, after LPS induction, increased approximately 50-fold. The levels of mRNAs for PDGF-A, M-CSF, and TNF increased 5 to 10-fold. However, the PDGF-B mRNA level remained constant for control and LPS-treated cells. Because the absolute amount of each mRNA was measured, this approach produces a detailed, yet multifaceted picture of the transcriptional phenotype in both the resting and the induced states using only fractions of micrograms of total RNA.

TABLE III

Specific mRNA levels (molecules/cell)* in LPS-Induced and Uninduced Human Macrophages**

mRNA Species	Uninduced	Induced	Induced/Uninduced
IL-1α	1.4	69	49
IL-1β	51	2,950	58
PDGF-A	0.05	0.48	10
PDGF-B	0.47	0.47	1
M-CSF	0.06	0.47	8
TNF	1.8	8.4	4.7

*Molecules/cell = molecules/μg RNA (calculated as in FIG. 2) $\times$ μg RNA isolated per cell.

**Monocyte-derived macrophages were cultured for ten days. 5 hours prior to harvest, half of the cultures were exposed to 5 μg/ml LPS.

EXAMPLE 5

Quantitative Analysis of Normal and Atherosclerotic Human Blood Vessels

Because accurate quantitative results can be obtained by the present technology even with small amounts of material, the method is an important tool for the analysis of samples which are rare or in limited quantity, e.g., in vivo-derived biopsy specimens. As an example, Table IV depicts the comparison of the results of quantitation of six different mRNA species from a human, atherosclerotic carotid artery and from a normal coronary artery. The data shows a 3- to 5-fold enhancement in the level of PDGF-A and PDGF-B mRNAs, no change in the type β PDGF receptor (PDGF-R) and a 3-fold decrease in the LDL receptor in the atherosclerotic vessel. There were increases in the levels of IL-1α and IL-1β mRNAs in the diseased tissue.

TABLE IV

Specific mRNA Levels (Molecules/μg Total RNA)* in a Normal and an Atherosclerotic Blood Vessel

mRNA Species	Atherosclerotic	Normal
PDGF-A	1.8×10^5	3.3×10^4
PDGF-B	7.6×10^4	2.2×10^4
PDGF-R	1.1×10^4	1.4×10^4
LDL-R	4.0×10^3	1.3×10^4
IL-1α	1.0×10^2	ND**
IL-1β	6.4×10^4	1.0×10^2

*Calculated as in FIG. 2.

**ND, Not Detectable

Other modifications of the embodiments of the invention described above that are obvious to those of ordinary skill in the areas of molecular biology, medical diagnostic technology, biochemistry, virology, genetics, and related disciplines are intended to be within the scope of the accompanying claims.

We claim:

1. A method for quantifying a target nucleic acid segment in a sample, which method comprises the steps of:

(a) adding to said sample a predetermined initial amount of standard nucleic acid segment wherein said standard nucleic acid segment binds to same primers as are bound by said target nucleic acid segment in a reaction mixture;

(b) treating said sample under conditions suitable for carrying out a polymerase chain reaction, wherein said nucleic acids are rendered single-stranded and exposed to an agent for polymerization, deoxynucleoside 5' triphosphates, and a pair of oligonucleotide primers, wherein said pair of primers is specific for both the target and standard nucleic acid segments, such that an extension product of each primer of said pair can be synthesized using separate strands of the target and standard segments as a template for synthesis, such that the extension product of one primer, when it is separated from the template strand, can serve as a template for the synthesis of the extension product of the other primer of said pair wherein said amplified target and standard segments are distinguishable by size or by the use of internal probes, wherein said internal probes may be differentially labeled for each of said amplified target and standard segments;

(c) separating the primer extension product from the templates on which they were synthesized to form single-stranded molecules;

(d) repeating steps (b) and (c) on the single stranded molecules produced in step (c) at least once, whereby each repeat of steps (b) and (c) is one amplification cycle;

(e) measuring the amounts of the amplified target and standard segments produced in step (d); and

(f) calculating from the amplified target and standard segments produced in step (d) the amount of said target nucleic acid segment present in the sample before amplification.

2. The method of claim 1 further comprising the step of preparing at least one dilution from the mixture of step (a) prior to step (b), whereby the amount of standard and target nucleic acid segments prior to said amplification reaction are varied.

3. The method of claim 1 wherein from 10 to 50 amplification cycles are performed, and wherein samples of said reaction mixture are removed and used to determine through which cycles the reaction is in exponential phase.

4. The method of claim 1 wherein said target nucleic acid segment is an mRNA molecule; said standard nucleic acid is a cRNA molecule; and said RNAs are reverse transcribed into cDNA molecules following step (a) and prior to step (b).

5. The method of claim 4 wherein the nucleic acid sequence of said cRNA molecule is synthesized using a plasmid template selected from the group AW108 and AW109.

6. The method of claim 4 wherein the pair of oligonucleotide primers of step (b) is labeled, and the amounts of amplified target and standard segments produced are measured according to step (e) by determining the amount of label incorporated into each of said amplified nucleic acid segments.

7. The method of claim 6 wherein said target nucleic acid segment is contained within an mRNA sequence, wherein said mRNA sequence encodes a lymphokine.

8. The method of claim 6 wherein said target nucleic acid segment is contained within a nucleic acid sequence, wherein said nucleic acid sequence encodes a protein selected from the group consisting of TNF, M-CSF, PDGF-A, PDGF-B, PDGF-R, apo-E, LDL-R, HMG, IL-1, IL-2, LPL, G-CSF, GM-CSF, aFGF, bFGF, c-fms, TGF-β, LFA-1, IL-2Rα, α-actin, desmin, β-actin, IL-6, IFN-α, IFN-γ, IL-6R, PDGF-αR, IL-2Rβ, IL-3, IL-4, and HIV proteins.

9. The method of claim 8 wherein said sample is a human blood cell sample or a human arterial vessel sample.

10. The method of claim 8 wherein said pair of oligonucleotide primers are selected from the group consisting of:

5'-CAGAGGGAAGAGTTCCCCAG-3',
5'-CCTTGGTCTGGTAGGAGACG-3';
5'-GAACAGTTGAAAGATCCAGTG-3',
5'-TCGGACGCAGGCCTTGTCATG-3';
5'-CCTGCCCATTCGGAGGAAGAG-3',
5'-TTGGCCACCTTGACGCTGCG-3';
5'-GAAGGAGCCTGGGTTCCCTG-3',
5'-TTTCTCACCTGGACAGGTCG-3';
5'-TTCCTGGCAGGATGCCAGGC-3',
5'-GGTCAGTTGTTCCTCCAGTTC-3';
5'-CAATGTCTCACCAAGCTCTG-3',
5'-TCTGTCTCGAGGGGTAGCTG-3';
5'-TACCATGTCAGGGGTACGTC-3',
5'-CAAGCCTAGAGACATAATCATC-3';
5'-GTCTCTGAATCAGAAATCCTTCTATC-3',
5'-CATGTCAAATTTCACTGCTTCATCC-3';
5'-AAACAGATGAAGTGCTCCTTCCAGG-3',
5'-TGGAGAACACCACTTGTTGCTCCA-3';
5'-GAATGGAATTAATAATTACAAGAATCCC-3',
5'-TGTTTCAGATCCCTTTAGTTCCAG-3';
5'-TGACCACCCAGCCATCCTTC-3',
5'-GAGGAGGTGTTGACTTCATTC-3'; and
5'-GAGATTTCTCTGTATGGCACC-3',
5'-CTGCAAATGAGACACTTTCTC-3'.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,219,727

DATED : June 15, 1993

INVENTOR(S) : Alice M. Wang, Michael V. Doyle, and David F. Mark

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Columns 7 and 8, Table I, under the section entitled "5' Primers", line 1,
please delete "5'-CAGAGGGAAGACTTCCCCAG-3'"
and insert therefor --5'-CAGAGGGAAGAGTTCCCCAG-3'--.

Columns 7 and 8, Table I, under the section entitled "3' Primers", line 4,
please delete "5'-TTTCTCACCTGGACAGGTGG-3'"
and insert therefor --5'-TTTCTCACCTGGACAGGTCG-3'--.

Columns 7 and 8, Table I, under the section entitled "3' Primers", line 9,
please delete "5'-TGTTTCAGATCCCTTTAGTTCCAG-3'"
and insert therefor --5'-TGGAGAACACCACTTGTTGCTCCA-3'--.

Columns 7 and 8, Table I, under the section entitled "3' Primers", line 10,
please delete "5'-TCCAGAACACCACTTGTTGCTCCA-3'"
and insert therefor --5'-TGTTTCAGATCCCTTTAGTTCCAG-3'--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,219,727
DATED : June 15, 1993
INVENTOR(S) : Alice M. Wang, Michael V. Doyle, and David F Mark

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Columns 7 and 8, Table I, under the section entitled "3' Primers", line 12,
please delete "5'-CTGCAAATGACACTTTCTC-3'"
and insert therefor --5'-CTGCAAATGAGACACTTTCTC-3'--.

Column 7, Table II, line 5, please delete "$^{5'}$TCCTTCCGGATGGCACAGTC$^{3'}$"
and insert therefor --$^{5'}$TCCTTCCGGATGGCACAGTG$^{3'}$--.

Signed and Sealed this

Sixth Day of September, 1994

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

US005476774A

United States Patent [19]

Wang et al.

[11] Patent Number: 5,476,774

[45] Date of Patent: * Dec. 19, 1995

[54] **QUANTITATION OF NUCLEIC ACIDS USING THE POLYMERASE CHAIN REACTION**

[75] Inventors: **Alice M. Wang**, Walnut Creek; **Michael V. Doyle**, Oakland, both of Calif.; **David F. Mark**, Plainsboro, N.J.

[73] Assignee: **Hoffmann-La Roche Inc.**, Nutley, N.J.

[*] Notice: The portion of the term of this patent subsequent to Jun. 15, 2010, has been disclaimed.

[21] Appl. No.: **28,464**

[22] Filed: **Mar. 9, 1993**

Related U.S. Application Data

[63] Continuation of Ser. No. 413,623, Sep. 28, 1989, Pat. No. 5,219,727, which is a continuation-in-part of Ser. No. 396, 986, Aug. 21, 1989, abandoned.

[51] **Int. Cl.**[6] **C12P 19/34**; C12Q 1/68; C12N 15/70; C07H 21/04

[52] **U.S. Cl.** **435/91.2**; 435/6; 435/320.1; 536/24.33; 935/77; 935/78

[58] **Field of Search** 435/6, 91.2, 320.1; 536/24.33; 935/77, 78

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,683,195 7/1987 Mullis et al. 436/6

OTHER PUBLICATIONS

Chelly et al, Nature, vol. 333, Jun. 30, 1988, pp. 858–860.

Primary Examiner—Mindy B. Fleisher
Attorney, Agent, or Firm—George M. Gould; Dennis P. Tramaloni; Stacey R. Sias

[57] **ABSTRACT**

The present invention provides a method for determining the amount of a target acid segment in a sample by polymerase chain reaction. The method involves the simultaneous amplification or the target nucleic acid segment and an internal standard nucleic acid segment. The amount of amplified DNA from each segment is determined and compared to standard curves to determine the amount of the target nucleic acid segment present in the sample prior to amplification. The method is especially preferred for determining the quantity of a specific mRNA species in a biological sample. Additionally, an internal standard is provided useful for quantitation of multiple mRNA species.

18 Claims, 4 Drawing Sheets

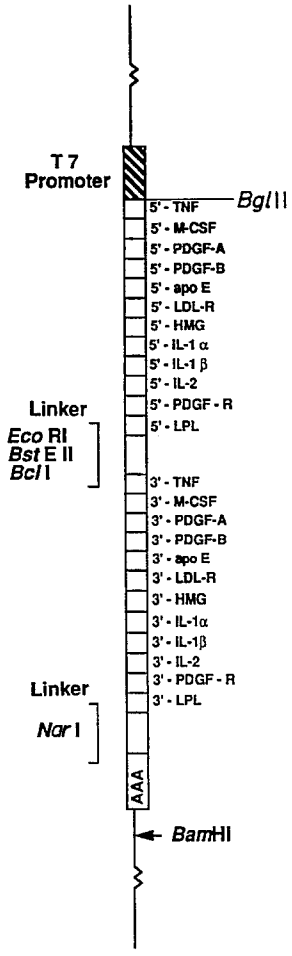


EXHIBIT O

FIG. 1

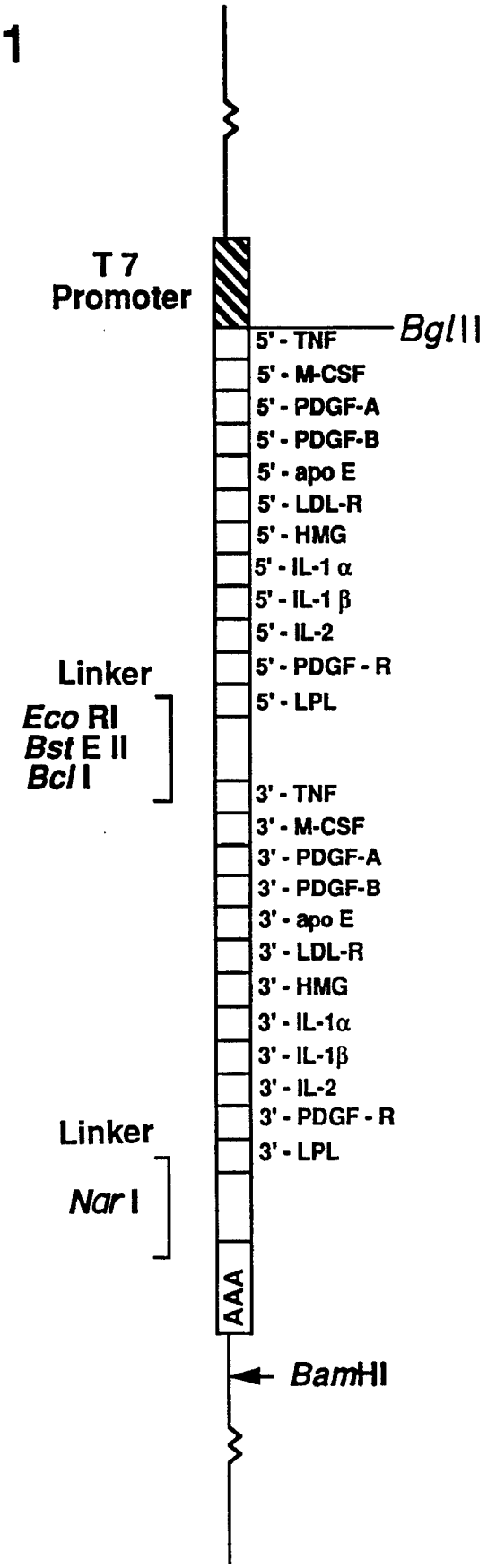

T 7
Promoter
Bgl II
5' - TNF
5' - M-CSF
5' - PDGF-A
5' - PDGF-B
5' - apo E
5' - LDL-R
5' - HMG
5' - IL-1 α
5' - IL-1 β
5' - IL-2
5' - PDGF - R
5' - LPL
Linker
Eco RI
Bst E II
Bcl I
3' - TNF
3' - M-CSF
3' - PDGF-A
3' - PDGF-B
3' - apo E
3' - LDL-R
3' - HMG
3' - IL-1α
3' - IL-1β
3' - IL-2
3' - PDGF - R
3' - LPL
Linker
Nar I
AAA
BamHI

FIG.2a

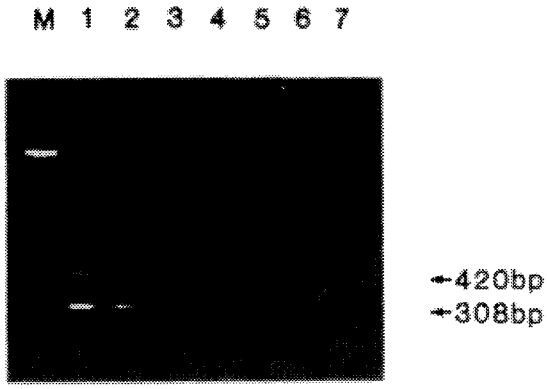


FIG.2b

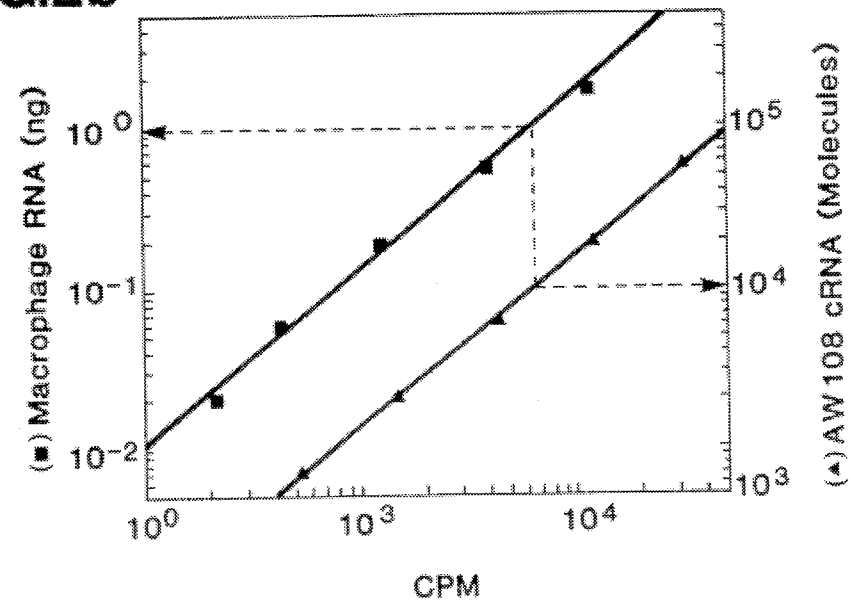


FIG.2c

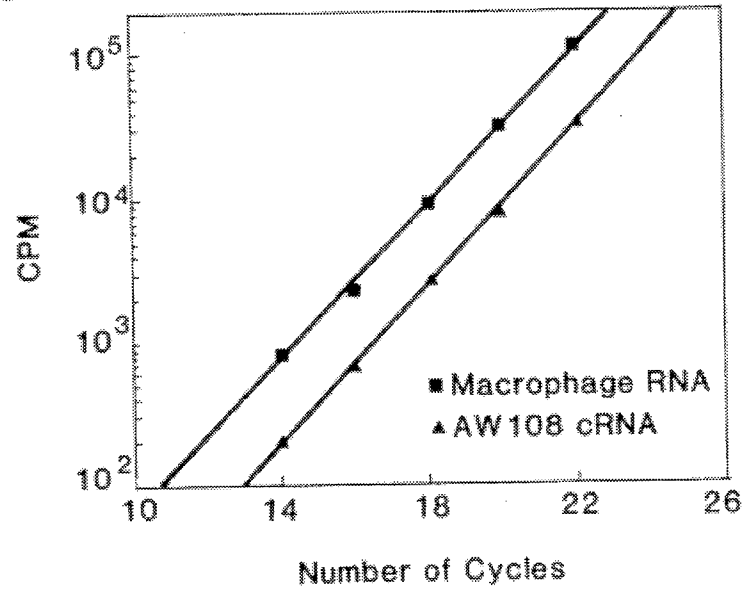
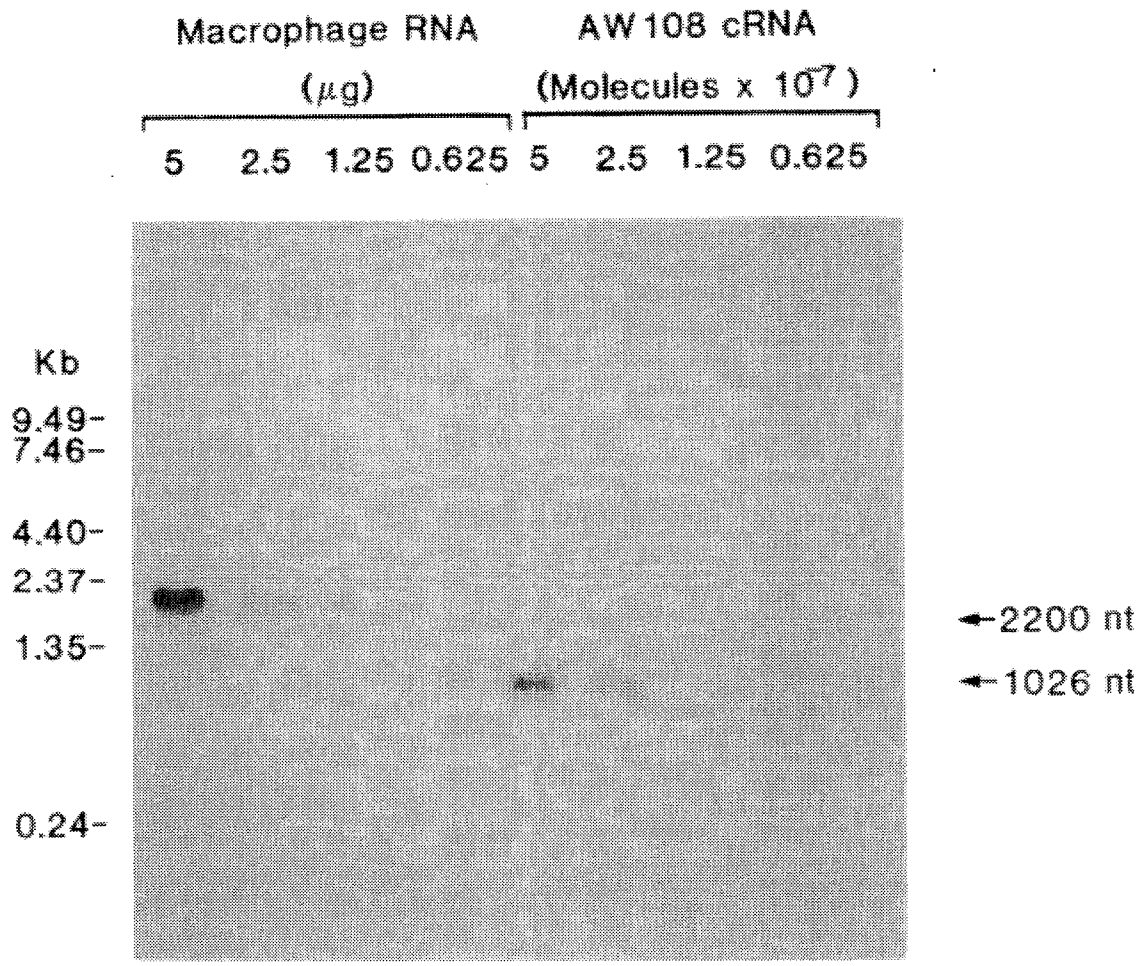


FIG.3


Macrophage RNA
(μg)
AW108 cRNA
(Molecules x 10^7)
5 2.5 1.25 0.625 5 2.5 1.25 0.625
Kb
9.49-
7.46-
4.40-
2.37-
1.35-
0.24-
2200 nt
1026 nt

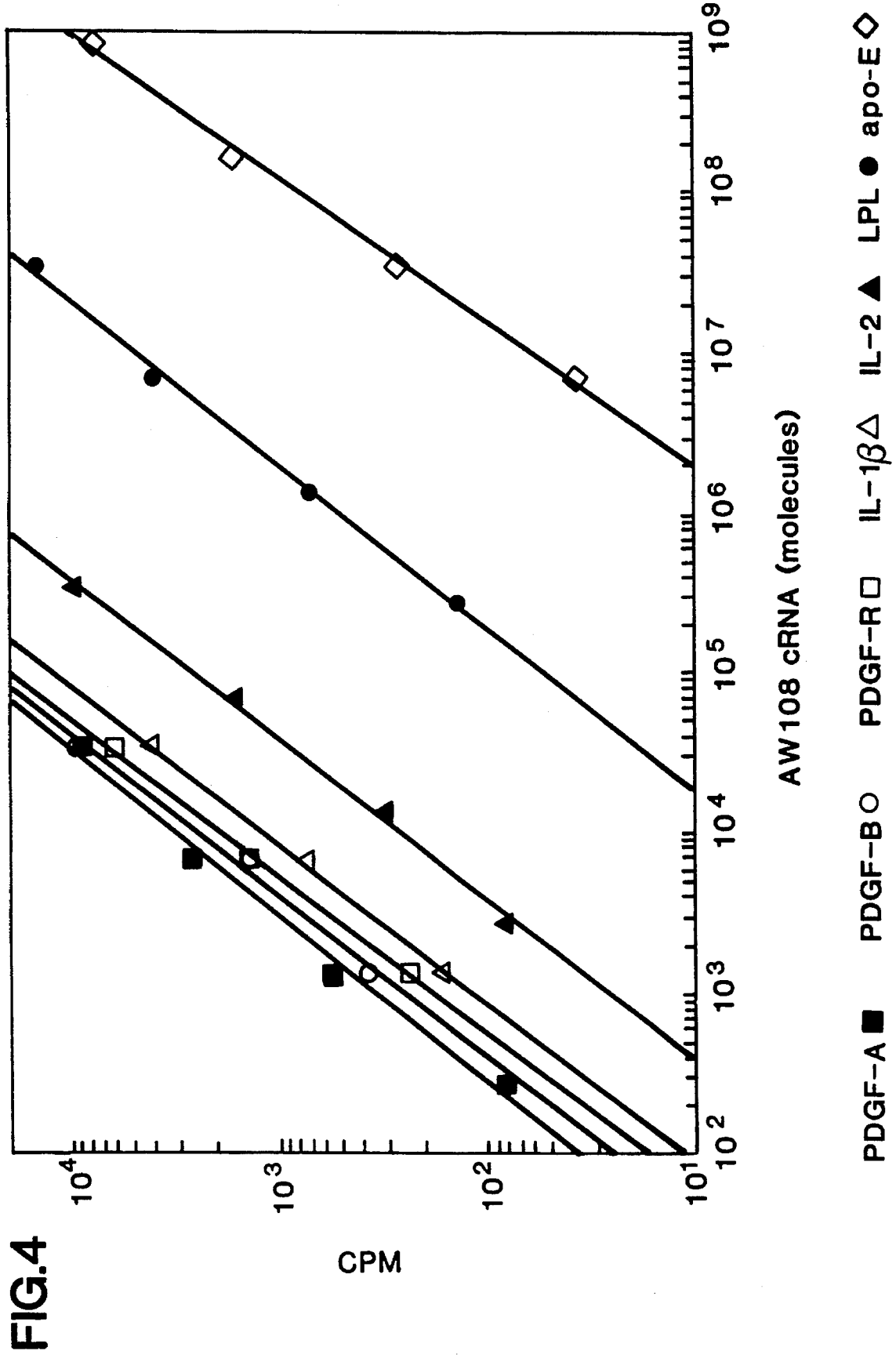

CPM
10^4
10^3
10^2
10^1
AW108 cRNA (molecules)
10^2
10^3
10^4
10^5
10^6
10^7
10^8
10^9
PDGF-A ■ PDGF-B ○ PDGF-R □ IL-1β △ IL-2 ▲ LPL ● apo-E ◇

FIG.4

QUANTITATION OF NUCLEIC ACIDS USING THE POLYMERASE CHAIN REACTION

CROSS REFERENCE TO RELATED APPLICATION

This application is continuation, of application Ser. No. 07/413,623, filed Sep. 28, 1989, now U.S. Pat. No. 5,219,727, which is a continuation-in-part of U.S. Ser. No. 396,986, filed Aug. 21, 1989, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the quantitative determination of a particular nucleic acid segment in a sample. The invention is particularly useful for determining the quantity of specific mRNA molecules in a biological sample. The method is therefore especially applicable in the field of medical diagnostics but can also be applied in the fields of genetics, molecular biology, and biochemistry.

2. Description of Related Disclosures

U.S. Pat. Nos. 4,683,195 and 4,683,702 disclose methods for carrying out the polymerase chain reaction (PCR), a nucleic acid amplification method, and for using PCR in the detection of specific nucleotide sequences. European Patent Office Publication (EPO) No. 258,017 describes Taq polymerase, a preferred DNA polymerase for use in PCR. These publications are incorporated by reference herein.

PCR methods have widespread applications in genetic disease diagnosis (see Wu et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:2757–2760 and Myerswitz, 1988, *Proc. Natl. Acad. Sci. USA* 85:3955–3959), as well as disease susceptibility and cancer diagnosis (see Horn et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:6012–6016; Todd et al., 1987, *Nature* 329:599–604; Kawasaki, 1988, *Proc. Natl. Acad. Sci. USA* 5698–5702; and Neri et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:9268–9272). However, these uses have provided only qualitative results by, for example, detecting unique mRNA transcripts from abnormal cells in a background of normal cells.

An attempt to use PCR for quantitative studies of mRNA levels for thymidylate synthase in tumors has been published (see Kashani-Sabet, 1988, *Cancer Res.* 48:5775– 5778). However, this study provides only relative comparisons of amounts of mRNA in biological samples. It has been much more difficult to quantitate the absolute amount of specific mRNA without an internal standard of known concentration. Other methods have been described for quantitating nucleic acid species by using PCR to co-amplify a second, unrelated, template cDNA (see Chelly et al., 1988, *Nature* 333:858–860 and Rappolee et al., 1988, *Science* 241:708–712). The use of an unrelated cDNA standard also necessitates the use of a second set of oligonucleotide primers, unrelated to the specific target mRNA.

Because amplification is an exponential process, small differences in any of the variables which control the reaction rate, including the length and nucleotide sequence of the primer pairs, can lead to dramatic differences in the yield of PCR product. Analyses which use two sets of unrelated primers, therefore, can only provide a relative comparison of two independent amplification reactions rather than an absolute measure of mRNA concentration.

Gilliland et al. (*J. Cellular Biochemistry*, UCLA Symposia on Molecular and Cellular Biology, Apr. 3–21, 1989, Abstract WH001) describe alternative approaches to mRNA quantitation to avoid some of the problems associated with unrelated templates as amplification standards. However, the Gilliland et al. suggestions have other inherent limitations. One approach requires mapping of genomic introns and exons for the gene corresponding to a specific target mRNA. Gilliland et al. also proposes an alternative approach using site directed mutagenesis to construct an internal standard, which causes the formation of heteroduplexes following amplification. These heteroduplexes result in an over estimation of the amount of target sequence present in the original sample. Smith et al. (Smith et al., 1989, *J. Immunol. Meth.* 118:265–272) have used an RNA dot blot assay to assess quantitatively the expression level of the two IL-1 mRNAs in human macrophages. Smith et al. reported that the level of sensitivity for IL-1α mRNA was approximately 10^7 molecules by his method, and IL- 1α mRNA was undetected in uninduced macrophages. The present invention provides a quantitation method which can readily measure 10^4 molecules and readily detects IL-1α mRNA in uninduced as well as induced macrophages in a sample assay. This 1000 fold increase in sensitivity represents an important advance in quantitative analysis for clinical and research purposes.

There remains a need for a method to quantitate directly, accurately, and reproducibly the amount of a specific nucleic acid segment in a sample. The availability of quantitative PCR will provide valuable information in a number of research areas. More particularly, the invention provides critical information in disease diagnosis and cancer therapy. For example, a reliable, sensitive, quantitative analysis can be critical in determining the extent of induction of mRNA synthesis in response to exogenous stimuli. The present invention overcomes the numerous limitations inherent in the attempts of others in this field, and thus provides means for accurately quantifying the amount of a nucleic acid segment in a biological sample.

SUMMARY OF THE INVENTION

The present invention provides a method for quantifying a target nucleic acid segment in a sample, which method comprises the steps of:

(a) adding to said sample an amount of standard nucleic acid segment;

(b) treating said sample under conditions suitable for carrying out a polymerase chain reaction, wherein said nucleic acids are rendered single-stranded and exposed to an agent for polymerization, deoxynucleoside 5' triphosphates, and a pair of oligonucleotide primers, wherein said pair of primers is specific for both the target and standard nucleic acid segments, such that an extension product of each primer of said pair can be synthesized using separate strands of the target and standard segments as a template for synthesis, such that the extension product of one primer, when it is separated from the template strand, can serve as a template for the synthesis of the extension product of the other primer of said pair;

(c) separating the primer extension product from the templates on which they were synthesized to form single-stranded molecules;

(d) repeating steps (b) and (c) on the single stranded molecules produced in step (c) at least once, whereby each repeat of steps (b) and (c) is one amplification cycle;

(e) measuring the amounts of the amplified target and standard segments produced in step (d); and

(f) calculating from the amplified target and standard segments produced in step (d) the amount of said target nucleic acid segment present in the sample before amplification.

The present invention also provides a plasmid useful for providing an internal standard for quantitation of target nucleic acid segments, said plasmid comprising a DNA sequence, said DNA sequence further comprising sequences which are identical to DNA sequences contained within said target nucleic acid segments.

The present invention also provides kits for the quantitation of specific nucleic acid segments in a biological sample.

DESCRIPTION OF THE FIGURES

FIG. **1** shows the positions of the 5' primers and 3' primers of Table I as they are arranged in plasmids AW108 and AW109. Other features are shown as they relate to the present invention.

In FIG. **2**A–C, the amount of IL-1α mRNA present in lipopolysaccharide (LPS) induced and uninduced macrophages was determined using the IL-1α primer pair.

FIG. **2**A depicts an ethidium bromide stained acrylamide gel wherein the amplified standard and target DNA segments are visible.

FIG. **2**B plots the amounts of standard and target IL-1α PCR product DNA produced against template concentrations.

FIG. **2**C shows a plot of the amounts of standard and template IL-1α PCR product DNA produced versus the number of amplification cycles.

FIG. **3** shows the results of a Northern blot containing samples of AW108 cRNA, and RNA isolated from LPS induced macrophages. The blot was probed with the IL-1α 3' primer.

FIG. **4** shows the efficiency of amplification for different primer sets using the same cRNA template under the same conditions.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for determining the absolute amount of a nucleic acid segment in a sample. The method involves amplification, by a polymerase chain reaction, of two different segments of nucleic acid combined in one reaction mix. The two segments include a target segment and an internal standard segment. The internal standard is amplified using the same oligonucleotide primer pair as the target nucleic acid; however, the two nucleic acid segments yield amplified products which are distinguishable by size.

The standard segment is present in a known amount. Following amplification, the amount of each of the two polymerase chain reaction products is measured, and the amount of the target segment present in the original sample is quantitated by extrapolating against a standard curve. In addition, the internal standard described herein contains primer sequences for multiple genes, so that the same standard can be used to quantitate a number of different nucleic acid segments of interest.

The present invention has particular utility in providing a rapid, sensitive, and reliable method for accurately determining the quantity of low abundance, specific mRNAs present in a sample containing less than 0.1 ng of total RNA. The method provides an approach powerful enough to enable a measurement of heterogeneity of expression levels of specific mRNAs within particular subpopulations even at the single cell level.

By co-amplification of the target nucleic acid and the internal standard nucleic acid, variable effects are internally controlled and affect the yield of PCR product equally for target and standard nucleic acids. Numerous variables influence the rate of the PCR reaction. Such variables may include the concentrations of polymerase, dNTPs, $MgCl_2$, nucleic acid templates, and primers, as well as the rate of "primer-dimer" formation and tube-to-tube variations.

The amount of the target nucleic acid segment present in the sample prior to amplification is determined using a standard curve. The standard curve is generated by plotting the amount of the standard segment produced in a polymerase chain reaction against varying, but known, amounts of the RNA present before amplification. For accuracy, the amount of standard segment present before amplification is varied by serial dilution of the co-amplification reaction mix. The amount of target segment produced in the polymerase chain reaction is then compared to the standard curve to determine the amount of target segment present in the sample prior to amplification. Alternatively, the standard curve may be generated by plotting the amount of standard and target segments produced against the number of amplification cycles. To ensure accuracy, it is preferred that the number of amplification cycles is varied by removing aliquots from one co-amplification reaction mixture after different numbers of amplification cycles have been completed.

The method of the invention is far superior to determinations of the amount of a nucleic acid segment in a sample as a relative, rather than absolute, amount. Further, the method is far more accurate than when an absolute amount is derived by employing a second set of oligonucleotide primers to amplify the standard, wherein that set of primers is different from the set used to amplify the target segment.

The method of the present invention is useful for quantifying a target RNA or DNA molecule. For determining an amount of DNA present in a sample, amplification methods described herein can be applied directly. As the examples disclosed below will demonstrate, the present invention is also useful in determining the amount of a specific mRNA in a sample of total RNA. The internal standard nucleic acid segment is provided on a DNA plasmid. The presence of an appropriately placed T7 polymerase promoter or another suitable promoter, such as the SP6 promoter, allows the plasmid to be used as a template for cRNA synthesis. As defined herein for the purpose of the present invention, the term "cRNA" refers to a ribonucleic acid segment synthesized from a DNA template by an RNA polymerase. Further, the plasmid may contain a polyadenylation sequence at the 3' end to facilitate purification and subsequently quantitation of the in vitro synthesized cRNA. As described in the preferred embodiments, the DNA template is either plasmid AW108 or AW109, and the RNA polymerase is T7 polymerase. In one embodiment AW108 cRNA is synthesized as a sense strand from pAW108 by T7 polymerase. The structure of pAW108 is shown in FIG. **1**. The primer array as shown in FIG. **1** is identical for both pAW108 and pAW109. The cRNA molecule then serves as the internal standard template for reverse transcription by the DNA polymerase, reverse transcriptase. Reverse transcriptase generates a cDNA transcript from an RNA template. The preferred embodiment of the invention, the internal standard cRNA, is synthesized as a sense strand. Following reverse transcrip-

tion of the target mRNA and the standard cRNA, PCR is then performed.

As will be obvious to those skilled in the art, numerous methods are known for constructing plasmids useful in the method of the present invention. Higuchi, 1988, *Nucleic Acids Research* 16:7351–7367 and Ho, 1989, *Gene* 77:51–59 describe two methods for engineering novel plasmids which incorporate desired synthetic DNA sequences. Alternatively, synthetic DNA segments can be inserted via restriction enzyme digestion and ligation with an appropriately treated parent plasmid or phage vector. The internal standard of the preferred embodiment, pAW108, contains multiple primer sets which allow a single cRNA standard to be used to quantitate a number of different mRNAs. The presence of unique restriction enzyme sites in the pAW108 plasmid provides the flexibility to add new primer sets to the plasmid. The unique BamHI site is used to linearize the plasmid to produce a linear template for reverse transcription. A deposit of *E. coli* containing plasmid AW108 has been deposited with the American Type Culture Collection (ATCC) at 12301 Parklawn Drive, Rockville, Md. according to the terms of the Budapest Treaty. A deposit of *E. coli* containing plasmid AW109 has also been deposited with the American Type Culture Collection (ATCC) at 12301 Parklawn Drive, Rockville, Md. according to the terms of the Budapest Treaty.

Plasmid AW108 is derived from pcDV1 and pL1 which are disclosed in Okayama and Berg, 1983, *Mol. Cell Biol.* 3:280–289. The SV40 promotor region from pLI was inserted into pcDV1 as directed in the referenced article. The T7 promoter, synthetic oligonucleotide sequences, and a polyadenylation region from the IL-1α gene were then inserted to provide the AW108 plasmid as an internal standard for the quantitation of twelve specific mRNAs. The plasmid was transformed into *E. coli* and grown in Luria Broth with ampicillin at 50 μl/ml added.

Plasmid AW108 was subsequently used as the starting material to construct pAW109. A culture of *E. coli* containing pAW108 was grown, and plasmid DNA was purified by standard means. The plasmid was digested with BamHI and BglII restriction endonucleases, and the 1 kb fragment was purified. This fragment contained the 5' and 3' primer arrays shown in FIG. **1** as well as the polyadenylation sequence. Plasmid pSP72 (Promega Biotec, Madison, Wis.) contains a T7 promoter adjacent to a polylinker to facilitate cloning. The plasmid also contains the ampicillin resistance gene.

The BglII-BamHI fragment from pAW108 was ligated into BglII and BamHI cleaved pSP72. Both of these are unique restriction sites within the polylinker region. The ligation mixture was used to transform *E. coli* DH5α, and resultant ampicillin resistant colonies were selected. The plasmid was assayed for the correct orientation of the BglII-BamHI insert. The resulting plasmid, pAW109, is suitable as an internal standard for mRNA quantitation.

As will be obvious to those skilled in the art, numerous other plasmids are available for insertion of desired DNA sequence to provide an internal standard useful in the present invention. Generally, the methods for transformation of such plasmids into a suitable host strain, propagation of the transformed host, and preparation of plasmid DNA as required for practice of the invention can be found in Maniatis et al., *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1985.

As used herein, the term "5' primer" refers to an oligonucleotide comprising a sequence identical to the sequence contained within the sense strand of a target nucleotide segment. As used herein, the term "3' primer" refers to an oligonucleotide comprising a sequence complementary to a sequence contained within the sense strand of the same target nucleotide segment. Thus, a 3' primer useful in the method of the present invention will hybridize to an mRNA, cRNA, or DNA template. It is further descriptive of the 3' and 5' primers that for both the internal standard cRNA and the target mRNA segment, the region of 3' primer hybridization is located 3' to the region of 5' primer hybridization.

The 3' and 5' primers function in the method of the present invention as follows: the 3' primer primes DNA synthesis in a PCR reaction to produce an anti-sense DNA strand, which provides a template for second strand DNA synthesis when the 5' primer is included in the PCR reaction. Such a 5' and 3' primer are referred to herein as a "primer pair."

In the preferred embodiment, most members of a primer pair are designed to span two exon-intron junctions within the gene encoding each target mRNA. In this way the primers will only hybridize effectively to the desired target mRNA. Thus, small amounts of contaminating genomic DNA in a biological sample will not effect accurate quantitation of the target mRNA.

Thus, a primer pair will function in a PCR reaction to amplify a segment of nucleic acid having a primer sequence identical to a DNA segment contained within the standard nucleic acid, i.e., as illustrated here, plasmids AW108 and AW109. As described herein, both plasmids contain a DNA sequence which comprises the DNA sequence of twelve primer pairs arranged as follows: DNA identical in sequence to the 5' primers of twelve target mRNAs is followed by the complementary DNA sequence of the 3' primers for the same twelve target mRNAs (FIG. **1**). The primer pair DNA sequence within pAW108 and pAW109 corresponds to mRNAs encoding tumor necrosis factor (TNF), macrophage-colony stimulating factor (M-CSF), platelet-derived growth factor A (PDGF-A), platelet-derived growth factor B (PDGF-B), low density lipoprotein receptor (LDL-R), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG), interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-2 (IL-2), type beta platelet-derived growth factor receptor (PDGF-βR), and lipoprotein lipase (LPL). The primer pairs useful for amplifying the internal standard provided by AW108 or AW109 cRNA in the practice of the method of the invention are depicted in Table I.

TABLE I

Oligonucleotides of 12 Target Genes' 5' Primers and 3' Primers

mRNA Species	5' Primers	3' Primers	Size of PCR Product (bp) mRNA	cRNA
TNF	5'-CAGAGGGAAGAMCCCCAG-3'	5'-CCTTGGTCTGGTAGGAGACG-3'	325	301
M-CSF	5'-GAACAGTTGAAAGATCCAGTG-3'	5'-TCGGACGCAGGCCTTGTCATG-3'	171	302
PDGF-A	5'-CCTGCCCATTCGGAGGAAGAG-3'	5'-TTGGCCACCTTGACGCTGCG-3'	225	301
PDGF-B	5'-GAAGGAGCCTGGGTTCCCTG-3'	5'-TTTCTCACCTOCACAGGTGG-3'	217	300
apo-E	5'-TTCCTGGCAGGATGCCAGGC-3'	5'-GGTCAGTTGTTCCTCCAGTTC-3'	270	301
LDL-R	5'-CAATGTCTCACCAAGCTCTG-3'	5'-TCTGTCTCGAGGGGTAGCTG-3'	258	301
HMG	5'-TACCATGTCAGGGGTACGTC-3'	5'-CAAGCCTAGAGACATAATCATC-3'	246	303
IL-1α	5'-GTCTCTGAATCAGAAATCCTTCTATC-3'	5'-CATGTCAAATTTCACTGCTTCATCC-3'	420	308
IL-1β	5'-AAACAGATGAAGTGCTCCTTCCAGG-3'	5'-TGGAGAACACCACTTGTTGCTCCA-3'	388	306
IL-2	5'-GAATGGAATTAATAATTACAAGAATCCC-3'	5'-TGTTTCAGATCCCTTTAGTTCCAG-3'	222	305
PDGF-R	5'-TGACCACCCAGCCATCTTC-3'	5'-GAGGAGGTGTTGACTTCATTC-3'	228	300
LPL	5'-GAGATTTCTCTGTATGGCACC-3'	5'-CTGCAAATGAGACACTTTCTC-3'	277	300

TNF, tumor necrosis factor, M-CSF, macrophage-colony stimulating factor; PDGF-A, platelet-derived growth factor A; PDGF-B, platelet-derived growth factor B; apo-E, apolipoprotein E; LDL-R, low density lipoprotein receptor; HMG, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IL-1α, interleukin-1α; IL-1β, intereukin-1β; IL-2, interleukin-2; PDGF-R, type β platelet-derived growth factor receptor; LPL, lipoprotein lipase.

Other mRNA targets which may be readily quantitated in biological samples by the present invention include, but are not limited to, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), acidic-fibroblast growth factor (aFGF), basic-fibroblast growth factor (bFGF), c-McDonough feline sarcoma (c-fms), transforming growth factor-β (TGF-β), leukocyte adhesion protein-1 (LFA-1), interleukin-2 receptor-α(IL-2Rα), alpha-actin, desmin, β-actin, interleukin-6 (IL-6), interferon-α (IFN-α), interferon-γ (IFN-γ), interleukin-6 receptor (IL-6R), platelet derived growth factor-α receptor (PDGF-αR), interleukin-2 receptor-β (IL-2Rβ), interleukin-3 (IL-3), and interleukin-4 (IL-4) as well as human immunodeficiency virus (HIV). Examples of primer pairs useful for the detection and measurement of expression of these RNAs are exemplified by the oligonucleotide sequences shown in Table II.

TABLE II

G-CSF	5' GGTGAGTGAGTGTGCCACCT 3', 5' GTTCTTCCATCTGCTGCCAG 3';
GM-CSF	5' CACTGCTGCTGAGATGAATGAAACAG 3', 5' GCACAGGAAGTTTCCGGGGTTGG 3';
aFGF	5' TCCTTCCGGATGGCACAGTG 3', 5' CATTTGGTGTCTGTGAGCCG 3';
bFGF	5' GACCCTCACATCAAGCTACAAC 3', 5' GGAAGAAAAGTATAGCTTTCTGC 3';
c-fms	5' CAAGTATAAGCAGAAGCCCAAGTAC 3', 5' GAGGGTCTTACCAAACTGCAGG 3';
TGF-β	5' CATCAACGGGTTCACTACCG 3', 5' TCCGTGGAGCTGAAGCAATAG 3';
LFA-1	5' GAGTGCCTGAAGTTCGAAAAGG 3', 5' CACACACTCTCGGCTCTCATC 3';
IL-2Rα	5' GCTGCCAGGCAGAGCTCTGTGACG 3', 5' GTTCCGAGTGGCAGAGCTTGTGC 3';
α-actin	5' GCACAACTGGCATCGTGCTG 3', 5' AGACTCCATCCCGATGAAGG 3';
desmin	5' AGGAGAGCCGGATCAACCTTC 3', 5' TCGCFGACGACCTCTCCATC 3';
β-actin	5' CCTTCCTGGGCATGGAGTCCTG 3', 5' GGAGCAATGATCTTGATCTTC 3';
IL-6	5' CCTTCTCCACAAGCGCCTTC 3', 5' GGCAAGTCTCCFCATTGAATC 3';
IFN-α	5' AGCTGCAAGTCAAGCTGCTC 3', 5' TCCCAAGCAGCAGATGAGTC 3';
IFN-γ	5' GAAGAATTGGAAAGAGGAGAGTGACAGAAA 3', 5' CATTCAAGTCAGTTACCGAATAATTAGTCAG 3';
IL-6R	5' CATTGCCATTGTTCFGAGGTTC 3', 5' AGTAGTCTGTATTGCTGATGTC 3';
PDGF-αR	5' CTGGATGAGCAGAGACTGAG 3', 5' AGGAAGCTGTCTTCCACCAG 3';
IL-2Rβ	5' TTTCAGGTGCGGGTCAAGCCTCTG 3', 5' AGTAACCCTGGTTGGTGAAGCAGC 3';
IL-3	5' CATGAGCCGCCTGCCCGTCC 3', 5' GGTTATTTTCCATCAGAATG 3';
IL-4	5' CTCACCTCCCAACTGCTTCCC 3', 5' GTGGAACTGCTGTGCAGTCGC 3'; and
HIV	5' AGTGGGGGGACATC 3',

TABLE II-continued

5' TTTGGTCCTTGTCTTATG 3'.

The PCR product from each primer set within pAW108 and pAW109 is 300–308 base pairs (bp), depending on the particular primer pair used. The 300–308 bp segment length of the illustrated example does not impose a limitation to the design of any internal standard. It is only necessary that the standard segment length is designed to be different in size from the PCR products of the target mRNAs and that the segment lengths be within the detection limits inherent in the analytical system preferred (for example, acrylamide gel electrophoresis, agarose gel electrophoreses, or other chromatographic means). The size difference between the PCR amplification products permits easy separation of the internal standard cRNA amplification product from the target mRNA amplification product by, for example, gel electrophoresis. The unique BamHI site is used to linearize the AW108 or AW109 plasmid to produce cRNA transcripts. Such transcripts are useful for quantitation of a number of different specific mRNAs in, for example, treated and untreated samples. This method can be used to provide a transcriptional phenotype of a treated or untreated cell or tissue and thus provides for numerous clinical and research applications.

The cRNA and the target mRNA are reverse transcribed in the same reaction. In this way, the cRNA serves not only as a standard for mRNA quantitation, but also provides an internal mRNA control for the reverse transcription reaction. Reverse transcriptase requires a primer to initiate cDNA synthesis using an RNA template. In the practice of the present invention, this will be an oligonucleotide primer which hybridizes to both the standard cRNA and the target mRNA. The primer may be identical in sequence to the 3' primer used for PCR amplification of that target mRNA. Alternatively, the primer for the reverse transcription reaction may be an oligonucleotide which hybridizes to the mRNA and cRNA at a position distal to the sequence of the 3' amplification primer, for example, oligo (dT). Thus, the resultant cDNA contains within it a sequence identical to the sequence of the 3' amplification primer. In the preferred embodiment disclosed herein, AW108 cRNA, as well as AW109 cRNA, contain a polyadenylation sequence at the 3' end, and oligo (dT) is used as a primer for reverse transcription of the cRNA and mRNA templates. Additionally, oligo (dT) permits amplification of more than one target sequence from the same reverse transcriptase reaction mix.

The same primers are used in the PCR amplification of both the standard and target templates; therefore, there are no primer efficiency differences between amplification of the standard and the target RNAs. When dilution series of mixtures of the target mRNA and internal standard cRNA are amplified in the same tube, and the reaction is terminated in the exponential phase of the amplification, the amount of target mRNA that was present in the sample prior to amplification can be determined by extrapolating against the internal standard cRNA standard curve. The amount of DNA produced is plotted against the amount of starting material for both the standard and the target. The standard curve allows extrapolation of the target data to determine the amount of target in the starting material. This value may be expressed as molecules of target mRNA/ng total RNA. Alternatively, it may be determined as of percentage or an amount by weight, or as a copy number.

Alternatively, a method is provided for determining the amount of target mRNA by varying the number of amplification cycles. The amount of amplified products produced is plotted against the number of amplification cycles for both the standard and target segments. The plotted data illustrates that portion of the reactions wherein the rate of amplification is exponential. Therefore, a ratio of products formed can be equated to a ratio of starting materials to determine the initial amount of target segment present. This is done according to the formula:

$$\frac{N_{o(mRNA)}}{N_{o(cRNA)}} = \frac{N_{(mRNA)}}{N_{(cRNA)}}$$

where N_o is the initial amount of material, and N is the amount of amplified product produced.

In another embodiment of the present invention, a third primer array is inserted into the internal standard plasmid between the 5' primer array and the 3' primer array. The third oligonucleotide array is comprised of a series of synthetic sequences wherein there is one sequence corresponding to each RNA for which the plasmid contains a 5' and 3' primer pair. This array is designed such that for each target RNA to be quantitated, the amplified product will contain within it a sequence identical to a portion of the third oligonucleotide array. Thus, both the amplified target and amplified standard DNA segments contain an identical internal segment providing a probe hybridization site, whereby for each primer pair, an oligonucleotide probe is useful to detect the amplified target as well as the amplified standard DNA.

Where a third oligonucleotide array is included in the standard plasmid, the PCR reaction can be carried out without the use of label. It is preferred that the reverse transcription and amplification reactions are carded out in separate tubes for each of the standard and target templates, rather than as a co-amplification. Following amplification, amount of product is quantitated by use of a dot blot format employing a single-stranded oligonucleotide probe which has a sequence corresponding to the internal sequence provided by the third primer array.

As an illustrative example of the present invention, the AW108 internal standard was used to determine the amount of several lymphokine mRNAs, including IL-1α mRNA, isolated from lipopolysaccharide (LPS)-induced and control cultures of human macrophages. Lymphokine mRNA levels were also measured in human atherosclerotic plaque tissue.

As provided by the present invention, target mRNA is quantified most accurately by using an internal standard having, in part, the same sequence as the target itself. Quantification of mRNA sequences by PCR amplification using an unrelated template as an internal standard provides only comparative data because of differences in efficiency between the primer pairs for the standard and the target mRNAs. This is inherent in the amplification process because PCR amplification is an exponential process. The extent of amplification (N) is given by the equation: $N=N_o(1+eff)^n$ where N_o is the initial amount of material, eff is the efficiency, and n is the cycle number. Thus, small differences in efficiency lead to large differences in the yield of PCR product and result in a misrepresentation of the amount of template present in a biological sample. Further, differences in primer efficiency are difficult parameters to

regulate for quantitative analyses. The present invention overcomes these problems.

The significant contribution of primer efficiency in the accurate quantitation of a nucleic acid segment is underscored in an example below. AW108 cRNA was used as the template for PCR amplification of several different primer sets. The efficiency of amplification by these different primer sets, under the same PCR conditions, varies over a range of several orders of magnitude. This invention addresses itself to this issue, which is clearly critical in any attempt to quantitate mRNA expression by PCR, and overcomes the problem of primer efficiency by using the same primers for amplification of the target mRNA and the internal standard cRNA.

The present invention requires that the amplification of the standard and target segments of nucleic acid be carried out in the same reaction. In the preferred embodiment of the present invention, the reverse transcriptase reaction of the standard cRNA and target mRNA is also carried out in the same reaction. Those skilled in the art will recognize from the foregoing that one could quantitate a target nucleic acid by performing the standard and target reverse transcriptase and amplification reactions separately. However, the accuracy of such a method is dependent on the degree to which the reverse transcription and amplification steps proceed with similar efficiency for both amplifications. By performing both reverse transcriptase reactions in the same tube and both amplification reactions in the same reaction tube, one ensures excellent accuracy.

The amount of an amplified DNA fragment in a given sample can influence amplification efficiency. When a high template concentration is used, or occurs as a result of the PCR amplification, phenomena can occur which are limiting factors for efficient amplification. Such phenomena include substrate saturation of enzyme, product inhibition of enzyme, incomplete product strand separation, and product strand reannealing. These problems are readily avoided, however, by an initial titration of the specific target mRNAs to find the range of concentrations that gives exponential amplification over a defined range of cycle numbers. Accordingly, to obtain reliable quantitative evaluation of specific mRNA using the described invention, the range of concentrations for both the standard and target templates, as well as the number of amplification cycles, should be such that the reactions remain within the exponential phase.

Thus, in the preferred embodiment, the reaction conditions described make use of 50 ng–1 μg of total cellular RNA combined with approximately 2×10^2–2×10^7 molecules of cRNA. As little as 50 pg cellular RNA is also suitable for purposes of the present invention. In the example described, as few as 1×10^4 molecules of IL-1α are detected. It is not necessary that mRNA be purified from a total RNA preparation in order to employ the method of the invention.

Samples suitable for analysis by this method may be of human or non-human origin; they may be derived from cultured samples, or isolated from dissected tissue or from cells of immunologically defined phenotype. The latter can be obtained by monoclonal antibody staining and fluorescence-activated cell sorter (FACS) isolation of enzyme-dissociated cells or by removal of specific areas from immunohistochemically stained slides. This will permit definitive identification of the cell types producing specific mRNAs.

The amount of amplified DNA generated in the method of the present invention can be measured in different ways. For instance, labeled primers wherein one or both members of any primer pair is labeled, or labeled nucleotides, can be used in PCR, and the incorporation of the label can be measured to determine the amount of amplified DNA. The label can be isotopic or non-isotopic. Alternatively the amount of amplified product can be determined by electrophoresis and visualization of the amplified product by staining or by hybridization with a labeled probe. Densitometry can be used to calculate the amount of product on a stained gel, or by extrapolation from an autoradiograph when labeled probe is used. When a labeled probe is used, the probe should be present in excess of the amplified product. In one such embodiment of the invention, primers are isotopically labeled and the resultant amplified products are electrophoresed on an acrylamide gel. The region where the product is expected to migrate is excised, and the amount of label present is determined by Cerenkov counting. The amount of label present is plotted versus the amount of known starting material.

The method of the invention requires that the amplified amounts of a template and standard segment produced in a single polymerase chain reaction be determined. Thus, the method requires that the amplified template segment be distinguishable from the amplified standard segment. If the segments are of different sizes, then it is relatively simple to distinguish one amplified segment from the other, i.e., the amplified products can be readily separated by gel electrophoresis. The present invention does not require that the amplified product be of different sizes, however, for other methods can be utilized to distinguish one amplified segment from another. For instance, the internal probe specific for one segment can be labeled differently than the internal probe specific for the other segment.

The quantitative method described herein is useful for analyses of in vivo biological samples. As is illustrated in the following example, quantitative PCR analysis of PDGF-A and B chain mRNA in a human atherosclerotic lesion versus a normal blood vessel emphasizes the sensitivity of this approach in investigating the biology of cells and tissues in vivo. For example, when the present method was used measure IL-1α and IL-1β mRNAs in atherosclerotic tissue, the results suggested that there may be inflammatory or immunological components in the pathogenesis of the disease.

Due to its high sensitivity, speed, and accuracy, the present quantitative PCR method can be used to study gene expression in a more extensive way than has been possible to date, allowing quantitative measurements of gene expression in a very small number of cells and from small amounts of tissue samples available from in vivo sources, such as biopsies. This technique can also provide information on changes in expression level of specific RNA molecules which may be valuable in the diagnosis and analysis of, for example, infectious disease states, cancer, metabolic disorders, and autoimmune diseases.

It will be apparent to those skilled in the art that the method of the present invention is amenable to commercialization as a kit for the quantitation of one or more nucleic acids in a sample. For example, in its simplest embodiment, such a kit would provide an internal standard and an appropriate oligonucleotide primer pair. In another embodiment, a kit may contain an internal standard, an appropriate oligonucleotide primer pairs, a DNA polymerase, a RNA polymerase, a reverse transcriptase, nucleotide triphosphates, restriction enzymes, buffers for carrying out cRNA and cDNA synthesis, restriction enzyme digests, and amplification by PCR. Further, the kits may contain a thermostable DNA polymerase; for example, the thermostable DNA polymerase Taq isolated from *Thermus aquaticus* as an agent of polymerization.

The method of the invention is exemplified below, but those skilled in the art will recognize the present invention is broadly applicable and in no way limited to the specific embodiments described below.

Example 1

Methods

A. Preparation of Internal Standard and RNAs

A synthetic gene was constructed using a technique of oligonucleotide overlap extension and amplification by PCR. The procedure used was similar to that described by Ho et al. for use in site-directed mutagenesis (Ho et al., 1989, *Gene* 77: 51–59). After construction, the synthetic gene was subcloned into an Okayama-Berg vector containing the T7 polymerase promoter and a polyadenylated sequence. The resulting plasmid, AW108, is shown in FIG. **1**. This plasmid was used as a template for transcription by T7 polymerase according to the transcription protocol of the manufacturer (Promega Biotec, Madison, Wis.). The resulting AW108 cRNA product was purified by oligo(dT) chromatography and gel electrophoresis. Alternatively, pAW109 was used to prepare a cRNA standard. The cRNA product was purified by selective elution using the Qiagen-tip system (Qiagen Inc., Studio City, Calif.) followed by oligo(dT) chromatography. The Qiagen-tip was used according to manufacturer's instructions for purification of RNA and run off RNA transcripts.

For either AW108 cRNA or AW109 cRNA, the purified cRNA was quantitated by determining absorbance at 260 nm. The number of molecules present was determined based on the molecular weight of the transcript. AW108 cRNA is 1026 nucleotides in length, therefore, 1 mole=3.386×10^5 gm (1026×330). Thus, 3.386×10^5 gm contains 6×10^{23} cRNA molecules. The number of molecules in 1 pg of AW108 cRNA is $(6\times10^{23})/(3.386\times10^5\ \text{gm})=1.77\times10^6$.

Total cellular RNA was isolated from macrophages and tissues by the method of acid guanidium thiocyanate-phenol-chloroform extraction according to Chomczynski et al., 1987, *Analyt. Biochem.* 162:156–159.

B. Purification of cRNA by Gel Electrophoresis

The cRNA prepared from pAW108 was electrophoresed in 1% low melt agarose, ultra pure grade, in TBE buffer. The region of the gel corresponding to 1 kb was cut out of the gel and melted in 0.2–0.4 ml of 0.1M NETS buffer (0.1M NaCl, 0.01M EDTA; 0.01M Tris-HCl, pH 7.4; 0.2% SDS) containing 1 mM 2-ME, in a water bath at 95° C. for 3–5 minutes and solidified quickly in an ice bucket. The samples were then frozen at −70° C. for at least two hours.

The frozen samples of melted agarose were thawed at 37° C. and centrifuged at top speed in an eppendorf centrifuge kept in the cold room. The agarose was pelleted out. The supernatant liquid was transferred to another eppendorf tube and extracted with a mixture of 100 µl phenol chloroform containing 1% isoamyl alcohol. The phenol was saturated with 0.1M NETS buffer. The aqueous phase was collected, and the RNA was ethanol precipitated. The RNA pellet was washed with 0.1 ml of 2M LiCl and then with 0.1 ml ethanol. The RNA was dried and then dissolved in an appropriate amount of sterile distilled water (2–100 µl) and was ready for reverse transcription.

C. Oligonucleotides Used for Amplification

Oligonucleotides were synthesized on a Biosearch (San Rafael, Calif.) DNA synthesizer. Most of the primers are RNA-specific primers. The 5' primers spanned the junction of the first two exons and the 3' primers spanned the junction of the next two exons. Alternatively, the 5' primers spanned the junction of the first and second exons and the 3' primers spanned the junction of the second and third exons. These sequences, the genes to which they correspond, and the sizes of amplified products obtained using the primers are shown in Table I.

D. cDNA Preparation

RNA was reverse transcribed into eDNA as previously described (see Gerard, 1987, *Focus* (Bethesda Research Labs) 9:5). A 10 µl reverse transcription reaction, containing 1 µg of total cellular RNA, 1.77×10^2–1.77×10^6 molecules of AW108 cRNA, 1×PCR buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µg/ml BSA), 1 mM DTT, 0.5 mM dNTP, 10 units RNasin (Promega Biotec), 0.1 µg oligo $(dT)_{12\text{-}18}$, and 100 units of BRL Moloney MuLV reverse transcriptase (Bethesda Research Laboratories) was prepared. The reaction was incubated at 37° C. for 60 minutes, heated to 95° C. for 5–10 minutes, then quickly chilled on ice.

E. Amplification Procedure

One tenth of the cDNA reaction mixture was diluted in a three-fold dilution series with 0.1 µg/µl tRNA, followed by adjustment to a final concentration of 1×PCR buffer, 50 µM dNTPs, 0.1 µM each of 5' and 3' primers, 1×10^6 cpm of ^{32}P end-labeled primer and 1 unit of Taq DNA polymerase (Perkin-Elmer Cetus) in a total volume of 50 µl. The mixture was overlaid with 100 µl mineral oil to prevent evaporation and then amplified for 25 cycles with the Perkin-Elmer Cetus Thermal Cycler. Alternatively, one tenth of the cDNA reaction mixture was amplified using the same conditions as above with varying numbers of cycles. The amplification profile involved denaturation at 95° C. for 30 seconds, primer annealing at 55° C. for 30 seconds, and extension at 72° C. for 1 minute. Oligonucleotides were labeled with γ-^{32}P-ATP by using polynucleotide kinase and unincorporated nucleotides were removed on a Bio-Gel P-4 column.

F. Quantitative Analysis

Ten µl of each PCR reaction mixture were electrophoresed in 8% polyacrylamide gels in Tris/borate/EDTA buffer. Gels were stained with ethidium bromide and photographed under UV-light irradiation. Appropriate bands were cut from the gel, and radioactivity was determined by Cerenkov counting. The amount of radioactivity recovered from the excised gel bands was plotted against the template concentrations. Data were plotted by exponential curve fitting with a Slide-Write Plus program (Advanced Graphics Software) The amount of target mRNA was quantitated by extrapolating against the AW108 cRNA internal standard curve.

G. Northern Blot Analysis

RNA was electrophoresed in a 1.5% agarose gel containing formaldehyde and transferred to a nitrocellulose filter in 20×SSC (1×SSC contained 0.15M sodium chloride and 0.015M sodium titrate). The blot was hybridized with 2×10^6 cpm of ^{32}P end-labeled oligonucleotides per ml. Hybridization was for 4 hours at 55° C. in 0.75M NaCl, 0.075M

sodium citrate, pH 7.0, 20 mM sodium phosphate, pH 7.0, 5 mM EDTA, 200 μg yeast RNA per ml, and 1% sarkosyl (Sigma). The blot was washed in 1×SSC at 55° C. for 30 minutes and autoradiographed with intensifying screens at −70° C.

H. Macrophage Cultures

Human peripheral blood monocytes were isolated from buffy coat preparations by Ficoll/Hypaque gradient centrifugation followed by adherence to plastic for one hour. Adherent cells were then removed and replated at 10^6 cells/well onto 6 well plates in RPMI 1640 medium supplemented with 2% fetal calf serum and 2000 units/ml recombinant macrophage-colony stimulating factor (Cetus Corporation). After ten days, half of the cultures were treated with 5 μg/ml LPS (Sigma). All the cultures were harvested for nucleic acid isolation 5 hours later.

I. Human Tissue Samples

The carotid endarterectomy sample was obtained during the course of a surgical operation with the informed consent of the patient. The RNA preparation of a histologically normal coronary artery was recovered from a heart transplant recipient.

Example 2

Quantification of IL-1α in a Preparation of Human Macrophage Total RNA

As an example of the present method, the AW108 internal standard was used to determine the amount of IL-1α mRNA isolated from LPS-induced cultures of human macrophages. Two different protocols were used to conduct this analysis. In the first case, the amount of template and standard RNAs was varied by serial dilution to generate a standard curve. In the second case, the number of amplification cycles was varied and plotted against the amount of PCR product.

A. Quantification of mRNA By Varying The Amount Of Internal Standard

Fifty ng of total macrophage RNA and 1.77×10^6 molecules of AW108 cRNA were combined and then reverse transcribed into cDNA. Serial three-fold dilutions of one tenth of the cDNA mixture were amplified using the IL-1α specific primers listed in Table 1. About 1×10^6 cpm of ^{32}P end-labeled 5' primer were included in the amplification. Reaction products were resolved by gel electrophoresis and visualized by ethidium bromide staining (FIG. **2**A). The amounts of radioactivity recovered from the excised gel bands were plotted against the template concentrations (FIG. **2**B). In this experiment, target mRNA and AW108 cRNA were amplified after serial three-fold dilutions, and the results demonstrate that the method can resolve less than three-fold differences in RNA concentrations. The fact that the reaction rates of AW108 cRNA and IL-1α mRNA amplification are identical within this exponential phase of the PCR reaction allows construction of a standard curve for AW108 cRNA and extrapolation to a copy number for the IL-1α mRNA present in the macrophages. As shown in FIG. **2**B, 1 ng of LPS-induced macrophage total RNA and 1×10^4 molecules of AW108 cRNA gave the same amount of IL-1α PCR product. In other words, 1 ng of LPS-induced macrophage RNA contained 1×10^4 molecules of IL-1α mRNA.

B. Quantification of mRNA by Varying The Number of Amplification Cycles

Five hundred ng of total macrophage RNA were reverse transcribed with 1.77×10^6 molecules of AW108 cRNA. Aliquots containing one tenth of the cDNA mixture each were subjected to 14, 16, 18, 20, 22, 24, 26, or 28 cycles of amplification under the same conditions as in protocol E. The amounts of radioactivity recovered from the excised bands were plotted as a function of the number of cycles (FIG. **2**C). The rates of amplification were exponential between 14 and 22 cycles for both templates. At 24, 26, and 28 cycles, the rates decreased drastically and approached a plateau. The efficiency of amplification was calculated from the slopes of these curves and found to be 88% for both AW108 cRNA and IL-1α mRNA. Because the amplification efficiency was the same for both co-amplified targets within the exponential phase, the absolute value of IL-1α mRNA can be calculated by comparison with the AW108 cRNA internal standard employing the formula:

$$\frac{N_{o(mRNA)}}{N_{o(cRNA)}} = \frac{N_{(mRNA)}}{N_{(cRNA)}}$$

where N_o is the initial amount of material, and N is the extent of amplification. The amount of IL-1α mRNA in 1 ng of LPS-induced macrophage total RNA calculated by this method was 1.1×10^4 molecules. Thus, the results using either of these two alternative protocols for quantitation are the same.

C. Correlation of PCR Results with Northern Analysis

The amount of IL-1α mRNA in LPS-induced macrophages determined by the quantitative PCR method was verified by Northern blot analysis. The PCR analysis (see above) demonstrated that 1 ng of macrophage RNA and 1×10^4 molecules of AW108 cRNA produced the same amount IL-1α PCR product. Thus, 5 μg of macrophage RNA and 5×10^7 molecules of AW108 cRNA should give similar signal intensities by Northern analysis. Two-fold serial dilutions of macrophage RNA and AW108 cRNA were subjected to Northern blot analysis by probing with the IL-1α 3' primer. The sizes of the target RNA molecules were estimated to be ~2,200 nucleotides for IL-1α mRNA in macrophages and 1026 nucleotides for AW108 cRNA. Hybridization signals of equal intensity were detected at all the dilutions of macrophage RNA and AW108 cRNA, as shown in FIG. **3**. This result demonstrates that the amount of mRNA estimated by the quantitative PCR method correlates with the results of Northern analysis.

Example 3

Effect of Primer Efficiency Differences

There are many variables which could influence the efficiency of the PCR amplification. Some of the parameters which can be controlled easily are the concentrations of template, dNTPs, $MgCl_2$, primers, polymerase, and PCR cycle profile. However, differences in primer efficiency are difficult parameters to regulate for quantitative analyses. To analyze the primer efficiency effect in the quantitative PCR method, AW108 cRNA was used as the template for PCR amplification of seven different primer sets: IL-1β, PDGF-A, PDGF-B, PDGF-R, IL-2, LPL, and apo-E. As indicated

in FIG. 4, the efficiency of amplification by these different primer sets under the same PCR amplification conditions varied over a range of several orders of magnitude. For instance, the IL-1β primers are 10^5-fold more efficient than the apo-E primers. Thus, it is critical to use the same primers for amplification of the target mRNA and the internal standard in any attempts to quantitate mRNA expression by PCR.

Example 4

Quantitation of Specific mRNAs in Untreated and LPS-Induced Macrophages

A major advantage of the present PCR quantitative technique is that the method enables one to analyze several target mRNA species in parallel. Table III shows the results from quantitation of the expression levels of six cytokine mRNAs in human macrophages in response to LPS treatment. The levels of IL-1β and IL-1α mRNAs, after LPS induction, increased approximately 50-fold. The levels of mRNAs for PDGF-A, M-CSF, and TNF increased 5 to 10-fold. However, the PDGF-B mRNA level remained constant for control and LPS-treated cells. Because the absolute amount of each mRNA was measured, this approach produces a detailed, yet multifaceted picture of the transcriptional phenotype in both the resting and the induced states using only fractions of micrograms of total RNA.

TABLE III

Specific mRNA levels (molecules/cell)* in LPS-Induced and Uninduced Human Macrophages†

mRNA Species	Uninduced	Induced	Induced/Uninduced
IL-1α	1.4	69	49
IL-1β	51	2,950	58
PDGF-A	0.05	0.48	10
PDGF-B	0.47	0.47	1
M-CSF	0.06	0.47	8
TNF	1.8	8.4	4.7

*Molecules/cell = molecules/μg RNA (calculated as in FIG. 2) × μg RNA isolated per cell.
†Monocyte-derived macrophages were cultured for ten days. 5 hours prior to harvest, half of the cultures were exposed to 5 μg/ml LPS.

Example 5

Quantitative Analysis of Normal and Atherosclerotic Human Blood Vessels

Because accurate quantitative results can be obtained by the present technology even with small amounts of material, the method is an important tool for the analysis of samples which are rare or in limited quantity, e.g., in vivo-derived biopsy specimens. As an example, Table IV depicts the comparison of the results of quantitation of six different mRNA species from a human, atherosclerotic carotid artery and from a normal coronary artery. The data shows a 3- to 5-fold enhancement in the level of PDGF-A and PDGF-B mRNAs, no change in the type β PDGF receptor (PDGF-R) and a 3-fold decrease in the LDL receptor in the atherosclerotic vessel. There were increases in the levels of IL-1α and IL-1β mRNAs in the diseased tissue.

TABLE IV

Specific mRNA Levels (Molecules/μg Total RNA)* in a Normal and an Atherosclerotic Blood Vessel

mRNA Species	Atherosclerotic	Normal
PDGF-A	1.8×10^5	3.3×10^4
PDGF-B	7.6×10^4	2.2×10^4
PDGF-R	1.1×10^4	1.4×10^4
LDL-R	4.0×10^3	1.3×10^4
IL-1α	1.0×10^2	ND †
IL-1β	6.4×10^4	1.0×10^2

*Calculated as in FIG. 2.
†ND, Not Detectable

Other modifications of the embodiments of the invention described above that are obvious to those of ordinary skill in the areas of molecular biology, medical diagnostic technology, biochemistry, virology, genetics, and related disciplines are intended to be within the scope of the accompanying claims.

We claim:

1. A plasmid for use as an internal standard for quantitation of at least one target nucleic acid sequence contained within a sample which plasmid comprises:

a standard nucleic acid segment comprising a 5' sequence and a 3' sequence which sequences provide upstream and downstream primer hybridization sites in said plasmid which primer hybridization sites are identical to upstream and downstream primer hybridization sites within said target nucleic acid sequence such that a primer pair, comprising an upstream oligonucleotide primer and a downstream oligonucleotide primer, will function in a PCR reaction to amplify said standard nucleic acid segment and said target nucleic acid segment, wherein upon amplification said standard and said target segments can be distinguished by size or by use of an internal oligonucleotide probe.

2. A plasmid according to claim **1** wherein said plasmid further comprises a polyadenylation sequence whereby said cRNA molecule can be used as a template in an oligo(dT) primed reverse transcriptase reaction.

3. A plasmid according to claim **1** that comprises the following DNA sequences:
5'-CAGAGGGAAGAGTTCCCCAG-3',
5'-CCTTGGTCTGGTAGGAGACG-3';
5'-GAACAGTTGAAAGATCCAGTG-3',
5'-TCGGACGCAGGCCTTGTCATG-3';
5'-CCTGCCCATTCGGAGGAAGAG-3',
5'-TTGGCCACCTTGACGCTGCG-3';
5'-GAAGGAGCCTGGGTTCCCTG-3',
5'-TTTCTCACCTGGACAGGTCG-3';
5'-TTCCTGGCAGGATGCCAGGC-3',
5'-GGTCAGTTGTTCCTCCAGTTC-3';
5'-CAATGTCTCACCAAGCTCTG-3',
5'-TCTGTCTCGAGGGGTAGCTG-3';
5'-TACCATGTCAGGGGTACGTC-3',
5'-CAAGCCTAGAGACATAATCATC-3';
5'-GTCTCTGAATCAGAAATCCTTCTATC-3',
5'-CATGTCAAATTTCACTGCTTCATCC-3';
5'-AAACAGATGAAGTGCTCCTTCCAGG-3',
5'-TGGAGAACACCACTTGTTGCTCCA-3';
5'-GAATGGAATTAATAATTACAAGAATCCC-3',
5'-TGTTTCAGATCCCTTTAGTTCCAG-3';
5'-TGACCACCCAGCCATCCTTC-3',
5'-GAGGAGGTGTTGACTTCATTC-3'; and
5'-GAGATTTCTCTGTATGGCACC-3',
5'-CTGCAAATGAGACACTTTCTC-3'.

4. A plasmid according to claim 3 wherein the plasmid is selected from the group consisting of pAW108 and pAW109.

5. A kit for the quantitation of a target nucleic acid segment in a biological sample comprising individual containers which provide:

a predetermined initial amount of an internal standard nucleic acid segment for quantitation of a target nucleic acid wherein said internal standard binds the same primers as are bound by said target nucleic acid segment; and

an oligonucleotide primer pair wherein said primer pair can serve to amplify said internal standard and said target nucleic acid.

6. The kit of claim 5 further comprising:

reverse transcriptase.

7. The kit of claim 5 wherein said target nucleic acid is contained within a nucleic acid sequence which encodes a protein selected from the group consisting of: TNF, M-CSF, PDGF-A, PDGF-B, apo-E, LDL-R, HMG, IL-1α, IL-β, IL-2, PDGF-R, LPL, G-CSF, GM-CSF, aFGF, bFGF, c-fms, TGF-β, LFA-1, IL-2Rα, α-actin, desmin, β-actin, IL-6, IFN-α, IFNγ, IL-6R, PDGF-αR, IL-2Rβ, IL-3, IL-4, and HIV proteins.

8. A plasmid according to claim 1, wherein said plasmid further comprises a T_7 polymerase promoter whereby a cRNA molecule can be produced using said standard nucleic acid segment as a template.

9. The plasmid of claim 1, wherein said target nucleic acid segment is contained within a nucleic acid sequence which encodes a protein selected from the group consisting of: TNF, M-CSF, PDGF-A, apo-E, LDL-R, HMG, IL-1α, IL-β, IL-2, PDGF-R, LPL, G-CSF, GM-CSF, aFGF, bFGF, c-fms, TGF-β, LFA-1, IL-2Rα, α-actin, desmin, β-actin, IL-6, IFN-α, IFN-γ, IL-6R, PDGF-αR, IL-2Rβ, IL-3, IL-4, and HIV proteins.

10. The kit of claim 5 further comprising a thermostable polymerase and appropriate buffers for a polymerase chain reaction.

11. The kit of claim 5, wherein said internal standard is provided by a DNA plasmid, wherein said DNA plasmid comprises a T_7 polymerase promoter whereby a cRNA molecule can be produced.

12. The kit of claim 6, wherein said internal standard is a cRNA molecule.

13. The kit of claim 11, wherein said DNA plasmid is selected from the group consisting of pAW108 and pAW109.

14. The kit of claim 12, wherein said cRNA is selected from the group consisting of pAW108 cRNA and pAW109 cRNA.

15. An amplification reaction mixture for the quantitation of a target nucleic acid segment in a biological sample, said reaction mixture comprising:

said target nucleic acid;

a predetermined initial amount of an internal standard nucleic acid segment for quantitation of a target nucleic acid, wherein said internal standard binds the same primers as are bound by said target nucleic acid segment; and

an oligonucleotide primer pair wherein said primer pair can serve to amplify said internal standard and said target nucleic acid, wherein following amplification said standard and target amplified nucleic acid segments are distinguishable by size or by use of internal hybridization probes.

16. The reaction mixture of claim 15, that further comprises a thermostable DNA polymerase and nucleoside triphosphates.

17. A reverse transcription reaction mixture for reverse transcribing a target mRNA suspected of being present in a biological sample, said reaction mixture comprising a predetermined initial amount of internal standard cRNA, a target RNA, and a target-specific primer for initiating cDNA synthesis, wherein said primer can serve to initiate reverse transcription of a nucleic acid segment contained within said standard cRNA together with a segment contained within the particular target nucleic acid, and wherein said standard segment is further distinguished by having a downstream hybridization site identical in sequence to a downstream by hybridization site in said target nucleic acid, whereby following reverse transcription the resulting target and standard cDNAs can serve as templates for amplification for providing standard and target amplified nucleic acid segments which are distinguishable by size or by use of internal hybridization probes.

18. The reaction mixture of claim 17 that further comprises a reverse transcriptase enzyme and nucleoside triphosphates.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,476,774

DATED : December 19, 1995

INVENTOR(S) : Alice M. Wang et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In column 18, Claim 2, line 2, after "whereby", please delete "said" and insert therefor --a--.

In column 20, Claim 17, line 11, after "downstream", please delete "by".

Signed and Sealed this

Seventh Day of May, 1996

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

United States Patent [19]

Gyllensten et al.

[11] **Patent Number: 5,066,584**

[45] **Date of Patent: Nov. 19, 1991**

[54] **METHODS FOR GENERATING SINGLE STRANDED DNA BY THE POLYMERASE CHAIN REACTION**

[75] Inventors: **Ulf B. Gyllensten**, Berkeley; **Henry A. Erlich**, Oakland, both of Calif.

[73] Assignee: **Cetus Corporation**, Emeryville, Calif.

[21] Appl. No.: **248,896**

[22] Filed: **Sep. 23, 1988**

[51] **Int. Cl.**[5] **C12P 19/34**

[52] **U.S. Cl.** **435/91**; 935/17

[58] **Field of Search** 435/91, 6; 536/27; 436/63, 94; 935/17

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,683,195	7/1987	Mullis et al.	435/6
4,683,202	7/1987	Mullis	435/91
4,800,159	1/1989	Mullis et al.	435/172.3
4,889,818	12/1989	Gelfand et al.	435/194
4,965,188	10/1990	Mullis et al.	435/6

FOREIGN PATENT DOCUMENTS

0258017	3/1898	European Pat. Off. .
0237362	9/1987	European Pat. Off. .

OTHER PUBLICATIONS

Fischer et al., Mar. 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:1579–1583.

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Scharf et al., 5 Sep. 1986, *Science* 233:1076–1078.

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Wrischnik et al., 1987, *Nuc. Acids. Res.* 15(2):529–542.

Mullis et al., 1987, *Methods Enzymol.* 155:335–350.

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Sakik et al., 29 Jan. 1988, *Science* 239:487–491.

Stoflet et al., 29 Jan. 1988, *Science* 239:491–494.

Scharf et al., May 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:3504–3508.

Horn et al., Aug. 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:6012–6016.

Gyllensten et al., Oct. 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:7652–7656.

Primary Examiner—James Martinell

Attorney, Agent, or Firm—Kevin R. Kaster

[57] **ABSTRACT**

Single stranded DNA can be generated by the polymerase chain reaction using two oligonucleotide primers, one persent in a limiting concentration. The single stranded DNA is useful in procedures involving utilizing nucleic acid probes and for purposes of nucleic acid sequencing.

7 Claims, 2 Drawing Sheets

FIG. 1 a

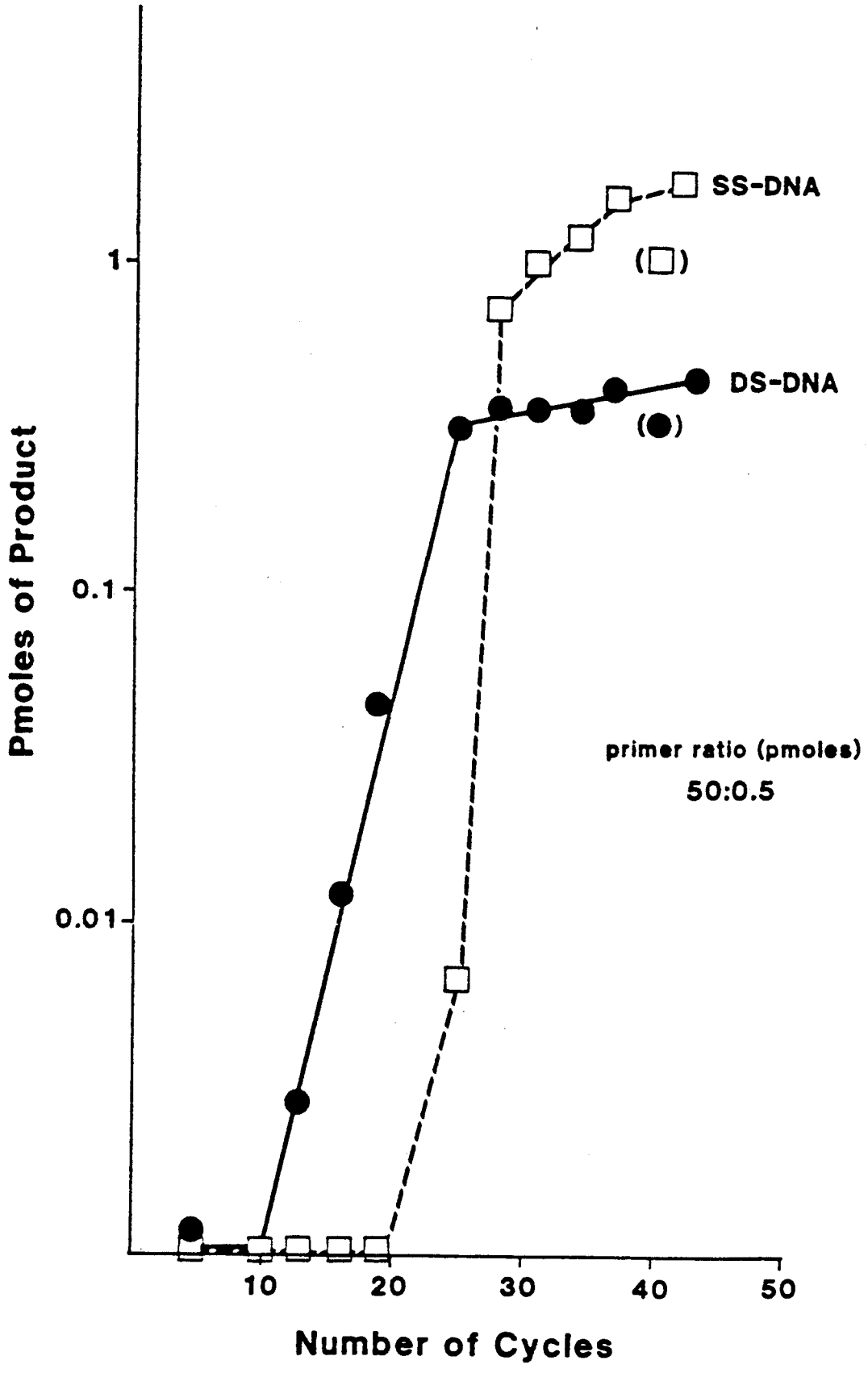
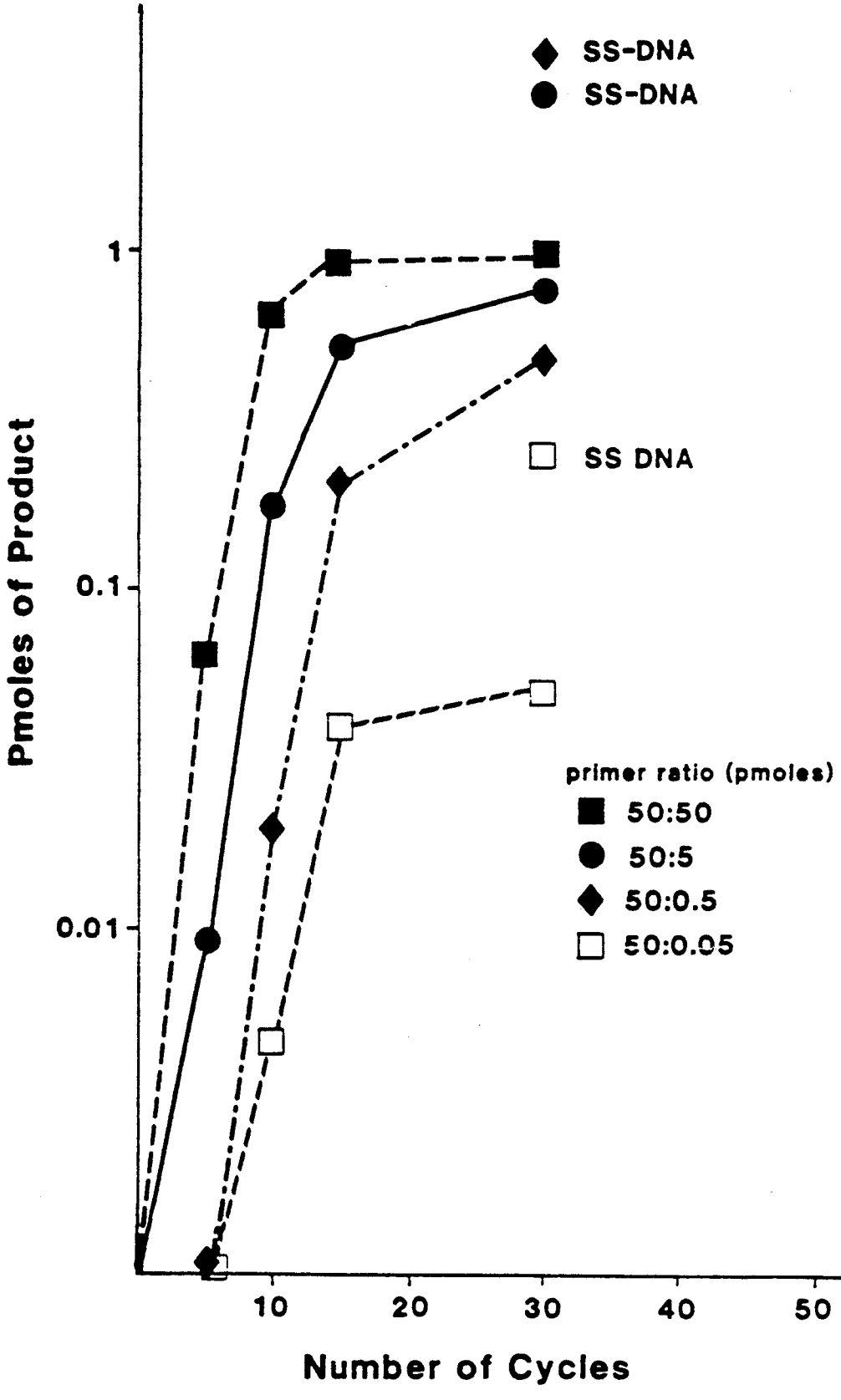

SS-DNA
(□)
DS-DNA
(●)
primer ratio (pmoles)
50:0.5
Pmoles of Product
1
0.1
0.01
10
20
30
40
50
Number of Cycles

FIG. 1 b


SS-DNA
SS-DNA
SS DNA
primer ratio (pmoles)
50:50
50:5
50:0.5
50:0.05
Pmoles of Product
1
0.1
0.01
10
20
30
40
50
Number of Cycles

METHODS FOR GENERATING SINGLE STRANDED DNA BY THE POLYMERASE CHAIN REACTION

BACKGROUND

1. Field of the Invention

Single stranded DNA plays a vital role in a number of different technologies. For instance, the determination of nucleic acid sequence has had an enormous impact in the fields of molecular biology, biochemistry, and genetics. Many sequencing protocols require utilization or generation of single stranded DNA at some stage of the process. The need for sequencing reagents and automated sequencing instruments has also created significant commercial activity. Single stranded nucleic acids are also used as probes in the emerging medical diagnostics technology based on hybridization of nucleic acids to detect pathogens and disease or disease-susceptible states. In addition, DNA "fingerprinting" techniques utilizing single stranded DNA probes has improved forensic methodologies.

2. Description of Related Disclosures

The present invention provides a method for generating single stranded DNA by the polymerase chain reaction (PCR). The PCR procedure involves repeated cycles of denaturation of the DNA, annealing of oligonucleotides primers to sequences flanking the segment of interest, and primer extension, typically mediated by a DNA polymerase, resulting in a doubling of the amount of a specific DNA fragment with each cycle. The PCR process requires a primer pair; the two primers define a segment of double stranded DNA that accumulates as the PCR product. The PCR process is described in U.S. Pat. No. 4,683,202 and results in a myriad number of identical copies of a double stranded DNA fragment.

PCR can be used to clone nucleic acid sequences never before identified or sequenced. The sequence of the PCR product can be identified either indirectly by hybridization to oligonucleotide probes, as described in U.S. Pat. No. 4,683,195, or directly by determination of the nucleotide sequence of the PCR amplified target. The sequence of DNA fragments generated by PCR has previously been determined either by cloning the fragments into derivatives of bacteriophage M13, as described by Scharf et al., 1986, *Science* 233:1076–1078, and Horn et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:6012–6016 or by direct sequencing of the double stranded template, using a third "internal" primer, as described by Wong et al., 1987, *Nature* 330:384–386, and Wrischnik et al., 1987, *Nuc. Acids Res.* 15:529–535. However, the M13 cloning method is time consuming and requires that several sequences be determined to distinguish mutations occurring in the original sequence from i) random point mutations introduced by lack of fidelity of the DNA polymerase; and ii) artifacts such as the formation of mosaic alleles by in vitro recombination. Direct sequencing of double stranded templates can present difficulties due to the rapid reannealing of strands and the presence of sequences partially homologous to that of the sequencing primer on both strands, resulting in compound sequence ladders. These problems can be overcome by the method of the present invention to modify the PCR reaction in such a way that an excess of full length single stranded DNA of a chosen strand is produced that is suitable for sequence determination.

SUMMARY OF THE INVENTION

The present invention provides a method for generating single stranded DNA by the polymerase chain reaction. The method, termed "asymmetric PCR", comprises:

(a) treating a nucleic acid with deoxyribonucleoside-5'-triphosphates, an agent for polymerization, and a pair of oligonucleotide primers under hybridizing conditions such that an extension product of a first primer of said primer pair is synthesized that is complementary to a nucleotide sequence in said nucleic acid, wherein the extension product of said first primer can serve as a template for synthesis of an extension product of a second primer of said pair;

(b) denaturing the extension products of said primers formed in step (a) from the templates on which they were synthesized; and

(c) treating the products of step (b) with the primers and under the conditions of step (a), wherein one of said first and second primers is present in limiting concentrations. The single stranded DNA generated in the reaction is the extension product of the primer present in the highest (not limiting) concentration during the reaction.

The present invention provides a number of significant advantages over known methods of producing single stranded DNA. The generation of single stranded DNA probes is a critical step in many important DNA sequencing methods and provides the basis for the diverse technologies that utilize nucleic acid probes and primers. The method is illustrated below in the direct sequencing of HLA DQα alleles in a heterozygote and for the generation of a nucleic acid probe. The present method has wide application in screening for and detecting mutations in genes and in facilitating automated DNA sequencing techniques.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** depicts accumulation of double and single stranded DNA during a polymerase chain reaction at different primer ratios. FIG. **1**(*a*) shows the result with a primer pair using a ratio of primer one:primer two of 50:0.5 pmol up to 43 cycles. FIG. **1**(*b*) shows the result with four different primer ratios: 50:50, 50:5, 50:0.5, and 50:0.05 pmol. The curves are based on densitometry scanning of autoradiographs.

DETAILED DESCRIPTION OF THE INVENTION

The present method for producing single stranded DNA by PCR requires that two amplication primers be present in different molar amounts.

The present invention provides a method for generating single stranded DNA by the polymerase chain reaction. The method, termed asymmetric PCR, comprises:

(a) treating a nucleic acid with deoxyribonucleaside-5'-triphosphates, an agent for polymerization, and a pair of oligonucleotide primers under hybridizing conditions such that an extension product of a first primer of said primer pair is synthesized that is complementary to a nucleotide sequence in said nucleic acid, wherein the extension product of said first primer can serve as a template for synthesis of an extension product of a second primer of said pair;

(b) denaturing the extension products of said primers formed in step (a) from the templates on which they

were synthesized; and (c) treating the products of step (b) with the primers and under the conditions of step (a), wherein one of said first and second primers is present in limiting concentrations. The single stranded DNA generating in the reaction is the extension product of the primer present in the highest concentration during the reaction.

This method generates single stranded DNA by a modified PCR. The PCR process is described in U.S. Pat. Nos. 4,965,188; 4,683,195; and 4,683,202 and can employ the thermostable polymerase described in U.S. Pat. No. 4,889,818. The PCR process has been automated; an apparatus capable of carrying out the reaction is disclosed in related copending Ser. No. 899,061, filed Aug. 22, 1986, which is a continuation-in-part of abandoned Ser. No. 833,368, filed Feb. 25, 1986. Methods for the structure-independent amplification of DNA by a PCR with the structure-destabilizing base analog 7-deazaguanine are described in Ser. No. 248,556, filed Sept. 23, 1988. Methods for dideoxy-sequencing single-stranded DNA utilizing the thermostable DNA polymerase, called Taq polymerase, from *Thermus aquaticus* are described in Ser. No. 249,367, filed Sept. 23, 1988. The disclosures of these related applications and patents are incorporated herein by reference.

The invention can be illustrated by a reaction in which two primers are present: 50 pmol of primer one and 0.5 pmol of primer two. During the first cycles, when the primer in limiting concentrations is still present, predominantly double stranded DNA will be produced. However, when most or all of the primer present in limiting concentrations has been extended by the reaction, an excess of single stranded DNA, the extension product of the primer present in excess, will be produced in each cycle. If the reaction were 100% efficient, after about 0.5 pmol of double stranded DNA (dsDNA) has been generated, single stranded DNA (ssDNA) will start to accumulate at a rate of 0.5 pmol per cycle of amplification. The resulting ssDNA can be sequenced either by adding more of the amplification primer initially present in limiting amounts, or by using an internal primer complementary to the ssDNA.

Theoretically, the amount of dsDNA should increase exponentially, whereas the production of ssDNA should only follow a linear growth. It is therefore important that the production of dsDNA is allowed to reach a certain level before ssDNA production is initiated. The optimal point for initiating the generation of ssDNA is a few cycles before the reaction has reached the level at which the amount of enzyme present in the reaction limits the growth in copy number. The overall efficiency of amplification when one primer is limiting appears somewhat lower (70%) as compared to when both are present in vast excess (80–90%, see Saiki et. al., 1988 *Science* 239:487–491). In practice, this decreased efficiency can usually be compensated for by increasing the number of PCR cycles.

The accumulation of double stranded DNA (as determined by densitometry scanning of autoradiographs) appears to follow the pattern predicted above with a short exponential phase of increase followed by a phase of linear growth, as shown in FIG. **1**(*a*). From the slope of the curve, the efficiency of amplification at various stages of the reaction can be calculated. During the exponential growth phase, the efficiency of accumulation of dsDNA is about 70%, while the efficiency of accumulation of ssDNA during the linear phase is only 30%. Practice of the present method with four different molar ratios of primers: 50:0.05, 50:0.5, 50:5, and 50:50 pmol, respectively, demonstrated that all three unequal molar ratios result in accumulation of ssDNA in amounts exceeding that of dsDNA after 30 cycles, as shown in FIG. **1**(*b*). Surprisingly, with a primer ratio of 50:5, about 0.8 pmol of dsDNA and several pmol of ssDNA are produced after 30 cycles, indicating that ssDNA is produced even under conditions where the limiting primers is not exhausted (4 pmol remaining). This is probably due to the fact that the reaction has reached a level where the amount of enzyme available is insufficient to completely extend all the templates present during each cycle. Although the amount of ssDNA generated will vary between primer sets, a ratio of 1:50–1:100 will, after 30 cycles of PCR, generally produce a sufficient excess of ssDNA (1 to 5 pmol) for several sequencing reactions.

The ssDNA generated by the method can be sequenced using eithe the PCR primer that is limiting or an internal primer capable of annealing to the ssDNA and then applying conventinal protocols for incorporation sequencing or labelled primer sequencing. The population of ssDNA strands produced should have discrete 5′ ends but may be truncated at various close to the 3′ end due to premature termination of extension. This 3′-end heterogenity will not affect sequencing of the ssDNA product, for only those ssDNA molecules that have been extended to include the sequence complementary to the sequencing primer will be utilized in the dideoxynucleotide sequencing reaction. Thus, the sequencing primer provides the discrete 5′ end for the product of the sequencing reaction. When the PCR primer present in limiting amounts is used as the sequencing primer, only full length ssDNAs can serve as templates in the sequencing reaction.

The developmemt of a simple PCR protocol for generating specific single strands significantly facilitates the direct sequencing of the amplified product, as well as the preparation of hybridization probes. The rapid identification of new mutants or allelic variants can be accomplished by amplifying DNA segments using locus specific primers. Similarly, as Saiki et al., describe, the analysis of unknown sequences can be carried out by amplifying a cloned insert using vector specific primers that flank the insertion site. In both cases, a single-tube reaction method for producing single stranded PCR products could be linked to an automated sequencing system for rapid sequence determination. The present invention provides this important single-tube reaction method.

This direct sequence procedure is also capable of identifying both alleles in a heterozygous individual. The resolution of two alleles that differ by more than two nucleotides requires allele specific oligonucleotides (ASOs) that are capable of either priming the sequencing reaction in an allele-specific fashion or amplifying by PCR in an allele-specific manner. Alternative approaches to the problem of sequencing heterozygotes are to use restriction enzymes to cleave one of the two alleles, as described by Scharf et al., 1988, *Proc. Natl. Acad. Sci. USA* 85: 3534–3508, or to use denaturating gradient gels to resolve the allelic PCR products, as described by Fisher et al., 1983, *Proc. Natl. Acad. Sci. USA* 80: 1579–1583. One virtue of direct sequence analysis over cloning of PCR products in M13 is the simultaneous of both alleles.

For example, HLA typing can be greatly facilitated by the present method to detect both alleles simulta-

neously. An individual serologically typed as DQw3 could either be homozygous DQw3/DQw3 or DQw3/blank (there are some "blank" alleles which are not reactive with (i.e., typed by) existing serologic reagents). Direct sequence analysis could immediately reveal the "hidden" blank allele. However, using prior art methods, one would have to sequence several different M13 clones before being able to distinguish the two potential genotypes. Another advantage of the present direct sequencing method is that low frequency, erroneous PCR products will not interfere with sequence determination. Point mutations occuring due to lack of fidelity of the DNA polymerase will, even if they arise in the very first cycle of amplification, only represent at most 25% of the intensity of the nucleotides of the consensus sequence when, for example, chain termination reactions such as those described by Innis et al., Ser. No. 249,367, for DNA sequencing are analyzed by gel electrophoresis. Occasionally, mosaic alleles have been observed in PCR mixtures, presumably resulting from partially extended PCR products that can act as primers on other allelic templates in later cycles. Such products are likely to accumulate primarily in later cycles of the reaction because of insufficient enzyme to extend all available templates. Unless such recombinant alleles arise frequently, however, the consensus sequence remains readily recognizable.

When the method of the present invention is utilized in a direct PCR sequencing protocol, such as that described by Innis et al., the analysis of allelic variants at a known locus as well as the determination of unknown sequences can be greatly facilitated. In conjunction with such sequencing procedures, the present method provides for rapid and simple analysis of nucleotide sequences, with particular benefit to manufacturers of automated DNA sequencing instruments.

Thus, the present method was used to sequence the HLA DQα locus of several homozygous typing cell lines. The sequence ladder obtained showed that only a single template is generated in the PCR reaction. Given the extensive polymorphism of HLA genes, most individuals are heterozygous at these loci. Because these alleles differ at multiple positions including small deletions, direct sequencing of HLA variants will be feasible only if individual alleles can be distinquish without cloning them apart. This is possible using allele specific oligonucleotides (ASO) for either: (1) sequencing of only one allele at a time in a mixture; or (2) amplification of only one of the two alleles.

For example, when a DR1/DR3 heterozygote individual was sequenced with one of the DQα PCR primers, a compound sequence was generated. The two alleles differed at several positions close to the primer and at a 3 base pair (3 bp) deletion. The sequences obtained using oligonucleotides specific for the DQA1 and DQA4.1 alleles, respectively, as sequencing primers were identical to that of the two alleles of the DR1/DR3; DQA1/DQA4.1 heterozygote. In general, only one of the two alleles needs to be sequenced separately, because by "subtracting" this sequence from the heterozygote sequence, the other allele can usually be identified. For example, the two alleles of a DQA4.1/-DQA3 individual differ by several nucleotide substitutions. Sequencing with a DQA4 ASO primer, one can identify one of the alleles, and the other allele can be subsequently reconstructed from the heterozygote sequence. It is also possible to use the ASOs to amplify selectively a specific allele in the heterozygote. These methods are suitable for sequencing alleles that are similar or identical to those previously described, but any difference between the ASO and the allele will be hidden and lost in the amplification. To identify the true genomic sequence to which the ASO anneals, a second oligonucleotide can be used as a sequencing primer.

The method of the present invention can also be used to generate radioactively labeled ssDNA, for use as a hybridization probe, as described in detail in Example 4. This aspect of the method of the invention was exemplified by amplifying a 242 bp segment of the HLA DQAα gene using primers flanking a hypervariable region. When the ratio of primer one to primer two was 50 pmol:0.5 pmol in PCR, the accumulation of product was observed over 43 cycles of amplification. A product of the expected size appeared, as detected by visibility on an ethidium bromide-stained gel, from about cycle 19. Under the conditions illustrated, accumulation appeared to stop at cycle 25. When a Southern blot of such a gel was probed with an internal oligonucleotide complementary to the ssDNA strand produced, the probe hybridized to the ssDNA band and a component with a lower molecular weight, corresponding to ssDNA. To confirm that the band represented ssDNA solely of one strand, the blot was stripped and reprobed with an oligonucleotide made to hyridize to the same strand as would the ssDNA. None of this probe hybridized to the band migrating at the molecular weight of ssDNA.

The above results were generated by using two oligonucleotide primers complementary to a central region of the 242 bp HLA DQAα fragment in the method of the invention. A plasmid containing DNA cloned from DQAα cDNA was used as the target nucleic acid. Thirty cycles of amplification were performed using three different molar ratios of primer one:primer two. These ratios were (a) 50:50, (b) 1:50 and (c) 50:1. The CPR reactions were performed in the presence of α-[^{32}P]dCTP. While reaction (a) only generated labeled dsDNA, both reactions (b) and (c) yielded ssDNA. The three amplifications were used as probes, without denaturation, to detect complementary sequences present in the product of a reaction analogous to reaction (b) but performed in the absence of radiolabeled nucleotide. Only the product of reaction (c) hybridized to both the ssDNA and dsDNA produced in the unlabeled (b) reaction. The product of reaction (b) only hybridized to the dsDNA, and the product of reaction (a) did not hybridize (as would be expected for undernatured dsDNA). These results demonstrate that the single stranded DNA produced by the present method is exclusively of one strand and can be utilized as a nucleic acid probe to detect specific nucleotide sequences.

Those skilled in the art recognize that the present method is suited to a variety of applications where the presence of single stranded DNA is desired. The present method is also preferred for use with other methodologies. For instance, the structure independent nucleic acid amplification method described by Innis et al. that utilizes 7-deaza-2′-deoxyguanosine-5′-triphosphate in PCR can also be used with the present method. The DNA sequencing method of Innis et al. can also be combined with the present invention to achieve a significant improvement over prior art methods. The following examples are provided merely to illustrate the invention and not to limit the scope of the accompanying claims.

EXAMPLE 1

Enzymatic Amplification of Genomic DNA

Genomic DNA (1 μg) was subjected to amplification with 2 units of the DNA polymerase from *Thermus aquaticus*, using conditions described in Saiki et al., 1988, *Science* 239:487–491, incorporated herein by reference. Thus, the reaction mixtures were in a volume of 100 μL and in a buffer composed of 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, each dNTP at 200 μM, and 200 μg/mL gelatin. The reaction mixture, including 50 pmol of primer GH26 (5'-GTGCTGCAGGTGTAAACTTGTACCAG) and 0.5 to 1 pmol of primer GH27 (5'-CACGGATCCG-GTAGCAGCGGTAGAGTTG) (Scharf et al., 1986, *Science* 233:1076–1078, incorporated herein by reference), were subjected to repeated PCR cycles of 30 seconds at 94° C., 1 minute at 55° C., and 2 minutes at 72° C. using a Perkin Elmer-Cetus Thermal Cycler. The reactions produced ssDNA complementary to a central region of the second exon of the DQα gene; the ssDNA produced was the extension product of the GH26 primer.

EXAMPLE 2

Electrophoretic Analysis of Amplifications

Aliquots of a reaction mixture prepared in substantial accordance with the procedure of Example 1 from successive cycles were dried down, resuspended in 5 μL of TE buffer, and electrophoresed through a 1% regular agarose, 3% NuSieve agarose gel for 2 hours at 5 volts/cm. Two μg of RF DNA from the phage φX174 cut with restriction enzyme HaeIII were used as a size standard. The agarose gel was then denatured for 45 minutes in a solution containing 0.4M NaOH and 1.5M NaCl, neutralized for 45 minutes in a solution containing 0.5M Tris-HCl, pH 7.5, and 1.5M NaCl, and the DNA transferred overnight in a solution of 4X SSC to a nylon filter (Genetran). The filter was baked for 1 hour at 80° C. in vacuum and prehybridized in a solution containing 5X SSPE, 5X Denhardt's solution, and 0.5% SDS for 15 minutes at 55° C.

Oligonucleotide probes specific for the DQα region were labeled with γ-^{32}P-ATP using T4 polynucleotide kinase. Unincorporated nucleotides were removed with the Centricon 30 microconcentrator (Amicon). Blots were hybridized in the same solution as that for the prehybridization, with addition of labeled oligonucleotide at a concentration of 0.1 pmol/mL, for 5 hours and then washed in 1X SSC at 42° C. for 30 minutes. The blots were exposed to Kodak X-omat film, and a number of different exposures were taken for densitometry scanning. The oligonucleotides were stripped off the blot by immersing the blot into distilled water at 60° C. for 15 minutes and prehybridized and rehybridized as above. This procedure, and the procedure of Example 1, modified as to primer ratios, was used to generate the data in FIGS. **1**(*a*) and **1**(*b*), which show accumulation of ssDNA and dsDNA in illustrative embodiments of the present invention as compared to when neither primer is present in limiting concentrations.

EXAMPLE 3

Sequencing of Single Stranded DNA

An entire amplification reaction (100 μL) produced in accordance with the procedure of Example 1 was mixed with 2 mL of distilled H_2O and applied to the Centricon 30 microconcentrator, spun at 5,000 rpm to remove excess dNTPs and buffer components, and the retenate collected in a volume of 40 μL. About 10 μL of the retenate were dried down and resuspended in 9 μL of 1X sequencing buffer (40 mM Tris-HCl, pH 7.5; 20 mM $MgCl_2$; and 50 mM NaCl) and 1 μL of a solution containing 10 μM sequencing primer. This resulted in an approximate primer to template ratio of 10:1. After the primer/template mixture was heated at 65° C. for 5 minutes, the primer was annealed to the template by dropping the temperature to 30° C. over a twenty minute period. The solution was then made 10 mM in DTT and 75 nM with respect to each of dGTP, dCTP, and TTP. Five μCi of α-[^{35}S]dATP (1000 Ci/mmol) and 2 units of modified T7 DNA polymerase (Sequenase ™, U.S. Biochemicals) were also added. The labeling reaction (16 μL) was continued for 5 minutes at room temperature and then aliquoted to each of four tubes with 2.5 μL of termination mix (each with 80 μM of each dNTP to 8 μM of the appropriate ddNTP). After 5 minutes of incubation at 37° C., the reaction was stopped by adding 4 μL of a solution of 95% formamide and 20 mM EDTA, heated to 75° C. for 2 minutes and loaded onto a 0.4 mm thick, 6% polyacrylamide/7M urea gel. The gel was run at 40 mA, 1.8 kV for 2.5 hours, fixed in 10% glacial acetic acid/10% methanol for 10 minutes, dried, and exposed to Kodak X-omat ™ film overnight. In general, this method produced sequencing ladders comparable to those generated using the same dideoxynucleotide sequencing procedure on single stranded M13 DNA.

EXAMPLE 4

Generation of Single Stranded Hybridization Probe

An 82 bp fragment, internal to the 242 bp HLA DQα product, was amplified using the primers GH84 and GH64. Fifty ng of a plasmid containing the DQα cDNA were subjected to 30 cycles of amplification using (a) 50 pmol of each primer, (b) 0.5 to 1.0 pmol of GH64 and 50 pmol of GH84, and (c) the reciprocal of (b). To obtain radiolabeled DNA, 5 μL of α-[^{32}P]dCTP (10μ Ci/μL, 1000 mCi/mmol) were added to the reaction, in addition to the nonradioactive nucleotides already present. Thus, during the amplification reaction by the present method (reactions (b) and (c)), ssDNA was generated that was labeled with ^{32}P and could thus serve as a probe preparation without denaturation. After amplification, the unincorporated nucleotides were removed using a Centricon microconcentrator. Another amplification reaction, analogous to (b) but without radiolabeled nucleotide, was electrophoresed and transferred to triplicate nylon membranes. Reactions (a), (b), and (c) were used to hybridize against the three nylon membranes. The results of the hybridization, discussed above, demonstrate that the present method can be used to produce ssDNA probes.

Other modifications of the embodiments of the invention described above that are obvious to those of ordinary skill in the areas of nucleotide chemistry, molecular biology, biochemistry, nucleic acid probe diagnostic technology, and related disciplines are intended to be within the scope of the accompanying claims.

We claim:

1. A method for generating single stranded DNA by the polymerase chain reaction, said method comprising:

(a) treating a nucleic acid with deoxyribonucleoside-5'-triphosphates, an agent for polymerization, and

first and second oligonucleotide primers under hybridizing conditions such that an extension product of said first primer is synthesized that is complementary to a nucleotide sequence in said nucleic acid, wherein the extension product of said first primer can serve as a template for synthesis of an extension product of said second primer and the first primer is present in excess of the second primer;

(b) denaturing the extension products of said primers formed in step (a) from the templates on which they were synthesized;

(c) treating the products of step (b) with the primers and under the conditions of step (a); and

(d) repeating steps (b) and (c) until said second primer is present in a limiting concentration so that the extension product of the first primer accumulates in excess of the extension product of the second primer.

2. The method of claim **1**, wherein said agent for polymerization is *Thermus aquaticus* DNA polymerase.

3. The method of claim **1**, wherein said primers are present in a ratio of first primer to second primer in the range of 10:1 to 1000:1.

4. The method of claim **1**, wherein said primers are present in a ratio of first primer:second primer of 10:1.

5. The method of claim **1**, wherein said primers are present in a ratio of first primer:second primer of 50:1.

6. The method of claim **1**, wherein said primers are present in a ratio of first primer:second primer of 100:1.

7. The method of claim **1**, wherein said primers are present in a ratio of first primer:second primer of 1000:1.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,066,584

DATED : November 19, 1991

INVENTOR(S) : H. Erlich and U. Gyllensten

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the cover sheet, under the heading "ABSTRACT", line 3, delete "persent" and insert therefor --present--.

Column 4, line 19, delete "eithe" and insert therefor --either--.

Column 4, line 24, after "various" insert --points--.

Signed and Sealed this

Sixteenth Day of March, 1993

Attest:

Attesting Officer

STEPHEN G. KUNIN

Acting Commissioner of Patents and Trademarks

United States Patent [19]

Gelfand et al.

[11] **Patent Number: 4,889,818**

[45] **Date of Patent: Dec. 26, 1989**

[54] **PURIFIED THERMOSTABLE ENZYME**

[75] Inventors: **David H. Gelfand,** Oakland; **Susanne Stoffel,** El Cerrito; **Frances C. Lawyer,** Oakland; **Randall K. Saiki,** Richmond, all of Calif.

[73] Assignee: **Cetus Corporation,** Emeryville, Calif.

[21] Appl. No.: **63,509**

[22] Filed: **Jun. 17, 1987**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 899,241, Aug. 22, 1986, abandoned.

[51] **Int. Cl.**[4] **C12N 9/00**

[52] **U.S. Cl.** **435/194;** 135/183; 935/14

[58] **Field of Search** 435/183, 194; 935/14

[56] **References Cited**

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4,683,195 7/1987 Mullis et al. .
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[57] **ABSTRACT**

A purified thermostable enzyme is obtained that has unique characteristics. Preferably the enzyme is isolated from the *Thermus aquaticus* species and has a molecular weight of about 86,000–90,000 daltons. The thermostable enzyme may be native or recombinant and may be used in a temperature-cycling chain reaction wherein at least one nucleic acid sequence is amplified in quantity from an existing sequence with the aid of selected primers and nucleotide triphosphates. The enzyme is preferably stored in a buffer of non-ionic detergents that lends stability to the enzyme.

3 Claims, 2 Drawing Sheets

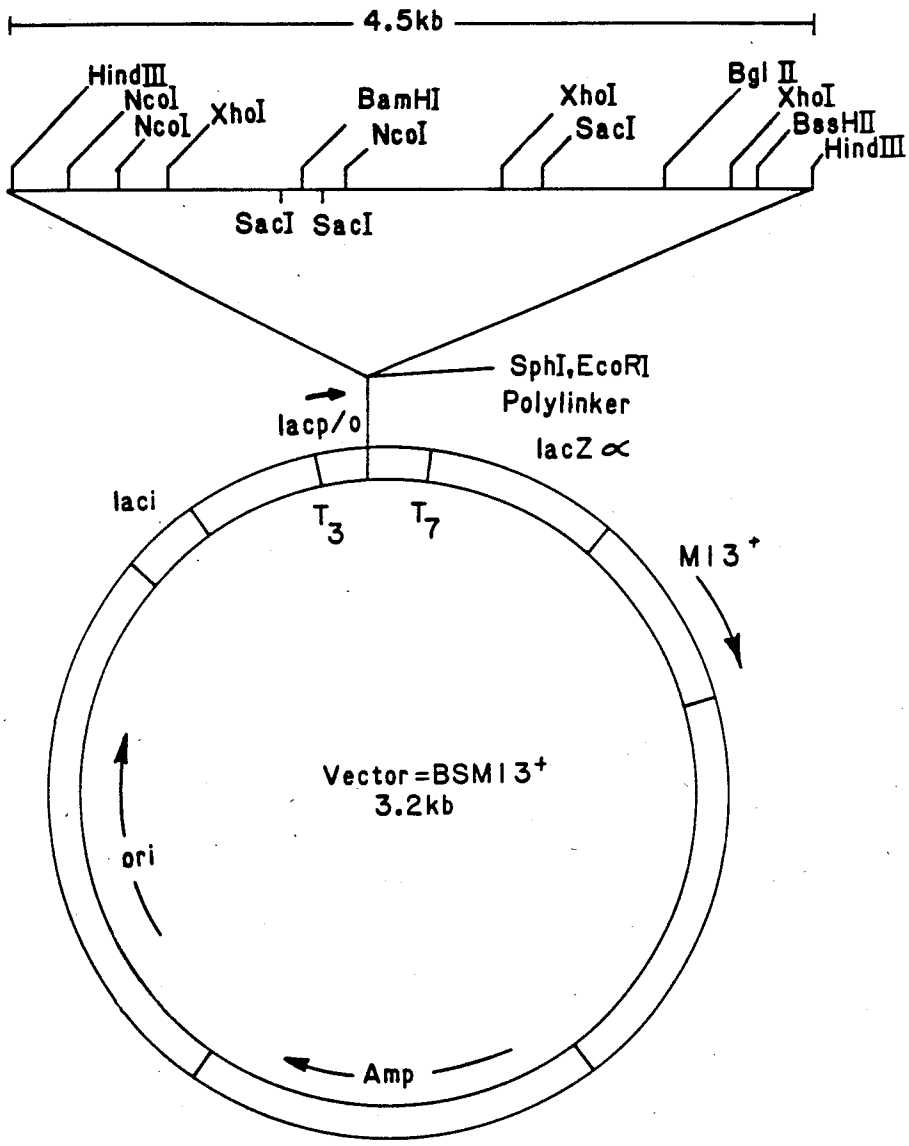
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FIG. 1

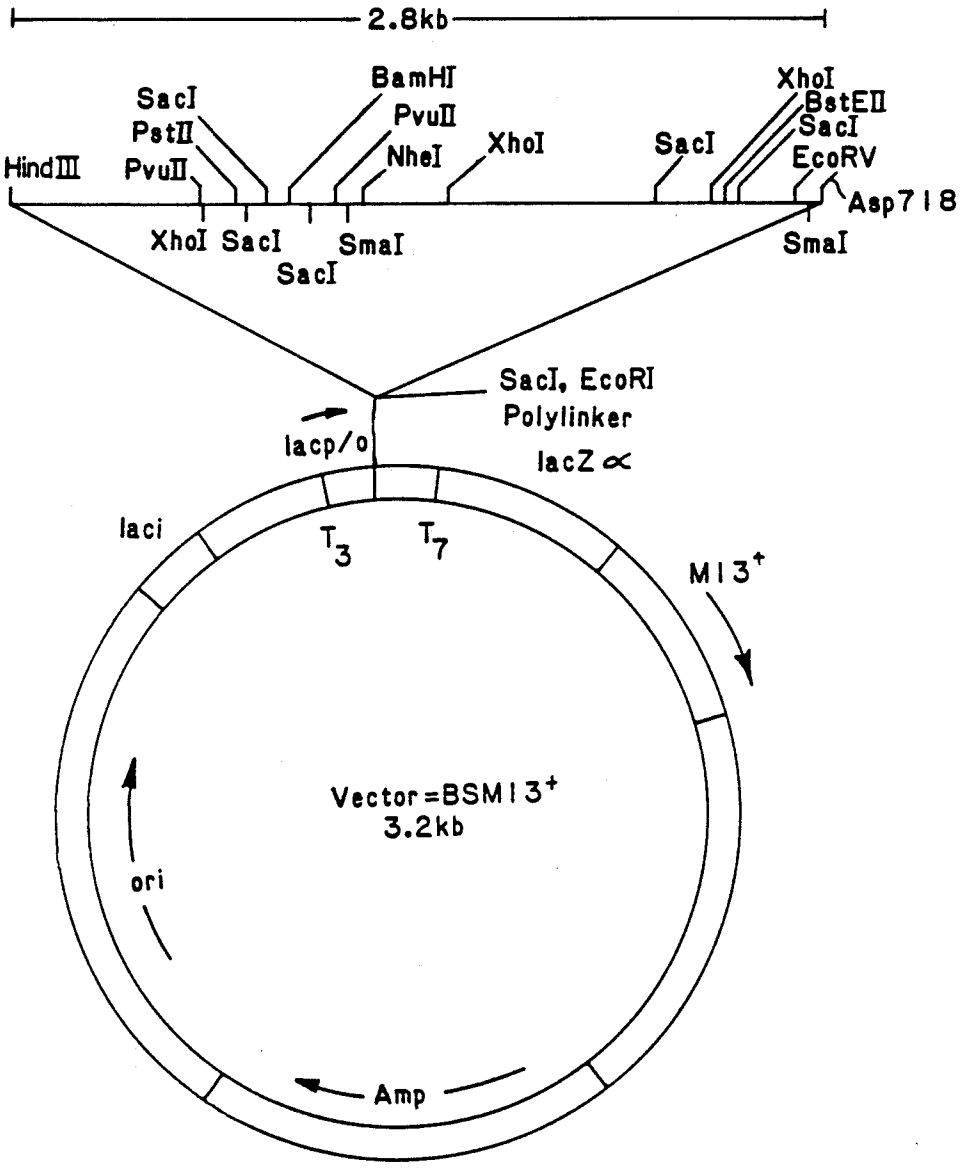
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FIG. 2

PURIFIED THERMOSTABLE ENZYME

CROSS-REFERENCE TO RELATED APPLICATION

This patent application is a continuation-in-part application of U.S. application Ser. No. 899,241, filed Aug. 22, 1986, now abandoned. This application is also related to copending U.S. application Ser. Nos. 899,344, filed Aug. 22, 1986, now abandoned which is a CIP of copending U.S. Ser. No. 839,331, filed Mar. 13, 1986, 899,061,filed Aug. 22, 1986, which is a CIP of copending U.S. Serial Nos. 833,368, filed Feb. 25, 1986; and 899,513, filed Aug. 22, 1986, which is a CIP of U.S. Pat. No. 4,683,195, filed Feb. 7, 1986, which is a CIP of U.S. Serial No. 824,044, filed Jan. 30, 1986, now abandoned, which is a divisional application of U.S. Pat. No. 4,683,202, filed Oct. 25, 1985, which is a CIP of U.S. Ser. No. 716,975, filed Mar. 28, 1985, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a purified thermostable enzyme. In one embodiment the enzyme is DNA polymerase purified from *Thermus aquaticus* and has a molecular weight of about 86,000–90,000.

2. Background Art

Extensive research has been conducted on the isolation of DNA polymerases from mesophilic microorganisms such as *E. coli.* See, for example, Bessman et al., *J. Biol. Chem.* (1957) 233:171–177 and Buttin and Kornberg (1966) *J. Biol. Chem.* 241:5419–5427.

In contrast, relatively little investigation has been made on the isolation and purification of DNA polymerases from thermophiles, such as *Thermus aquaticus.* Kaledin et al., *Biokhymiya* (1980) 45:644–651 discloses a six-step isolation and purification procedure of DNA polymerase from cells of *T. aquaticus* YT1 strain. These steps involve isolation of crude extract, DEAE-cellulose chromatography, fractionation on hydroxyapatite, fractionation on DEAE-cellulose, and chromatography on single-strand DNA-cellulose. The pools from each stage were not screened for contaminating endo- and exonuclease(s). The molecular weight of the purified enzyme is reported as 62,000 daltons per monomeric unit.

A second purification scheme for a polymerase from *T. aquaticus* is described by A. Chien et al., *J. Bacteriol.* (1976) 127:1550–1557. In this process, the crude extract is applied to a DEAE-Sephadex column. The dialyzed pooled fractions are then subjected to treatment on a phosphocellulose column. The pooled fractions are dialyzed and bovine serum albumin (BSA) is added to prevent loss of polymerase activity. The resulting mixture is loaded on a DNA-cellulose column. The pooled material from the column is dialyzed and analyzed by gel filtration to have a molecular weight of about 63,000 daltons, and, by sucrose gradient centrifugation of about 68,000 daltons.

The use of a thermostable enzyme to amplify existing nucleic acid sequences in amounts that are large compared to the amount initially present has been suggested in copending U.S. Pat. No. 4,683,195. Primers, nucleotide triphosphates, and a polymerase are used in the process, which involves denaturation, synthesis of template strands and hybridization. The extension product of each primer becomes a template for the production of the desired nucleic acid sequence. The application discloses that if the polymerase employed is a thermostable enzyme, it need not be added after every denaturation step, because the heat will not destroy its activity. No other advantages or details are provided on the use of a purified thermostable DNA polymerase. Furthermore, New England Biolabs had marketed a polymerase from *T. aquaticus,* but discovered that the polymerase activity decreased substantially with time in a storage buffer not containing non-ionic detergents.

Accordingly, there is a desire in the art to produce a purified, stable thermostable enzyme that may be used to improve the diagnostic amplification process described above.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a purified thermostable enzyme that catalyzes combination of nucleotide triphosphates to form a nucleic acid strand complementary to a nucleic acid template strand. Preferably the purified enzyme is DNA polymerase from *Thermus aquaticus* and has a molecular weight of about 86,000–90,000 daltons. This purified material may be used in a temperature-cycling amplification reaction wherein nucleic acid sequences are produced from a given nucleic acid sequence in amounts that are large compared to the amount initially present so that they can be detected easily.

The gene encoding the enzyme from DNA polymerase from *Thermus aquaticus* has also been identified and provides yet another means to retrieve the thermostable enzyme of the present invention. In addition to the gene encoding the approximately 86,000–90,000 dalton enzyme, gene derivatives encoding DNA polymerase activity are also presented.

Finally, the invention also encompasses a stable enzyme composition comprising a purified, thermostable enzyme as described above in a buffer containing one or more non-ionic polymeric detergents.

The purified enzyme, as well as the enzymes produced by recombinant DNA techniques, provide much more specificity than the Klenow fragment, which is not thermostable. In addition, the purified enzyme and the recombinantly produced enzymes exhibit the appropriate activity expected when dTTP or other nucleotide triphosphates are not present in the incubation mixture with the DNA template. Also, the enzymes herein have a broader pH profile than that of the thermostable enzyme from *Thermus aquaticus* described in the literature, with more than 50% of the activity at pH 7 as at pH 8. Finally, the thermostable enzyme herein can be stored in a buffer with non-ionic detergents so that it is stable, not losing activity over a period of time.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a restriction site map of plasmid pFC83 that contains the ~4.5 kb HindIII *T. aquaticus* DNA insert subcloned into plasmid BSM13+.

FIG. 2 is a restriction site map of plasmid pFC85 that contains the ~2.8 kb HindIII to Asp718 *T. aquaticus* DNA insert subcloned into plasmid BSM13+.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein, "cell", "cell line", and "cell culture" can be used interchangeably and all such designations include progeny. Thus, the words "transformants" or "transformed cells" includes the primary subject cell

and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for procaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eucaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

The term "expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. In order to effect transformation, the expression system may be included on a vector; however, the relevant DNA may then also be integrated into the host chromosome.

The term "gene" as used herein refers to a DNA sequence that encodes a recoverable bioactive polypeptide or precursor. The polypeptide can be encoded by a full-length gene sequence or any portion of the coding sequence so long as the enzymatic activity is retained.

"Operably linked" refers to juxtaposition such that the normal function of the components can be performed. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequences can be expressed under the control of the sequences.

"Non-ionic polymeric detergents" refers to surface-active agents that have no ionic charge and that are characterized, for purposes of this invention, by their ability to stabilize the enzyme herein at a pH range of from about 3.5 to about 9.5, preferably from 4 to 8.5.

The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide. The oligonucleotide may be derived synthetically or by cloning.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of four different nucleotide triphosphates and thermostable enzyme in an appropriate buffer ("buffer" includes pH, ionic strength, cofactors, etc.) and at a suitable temperature. For Taq polymerase the buffer herein preferably contains 1.5–2 mM of a magnesium salt, preferably $MgCl_2$, 150–200 μM of each nucleotide, and 1 μM of each primer, along with preferably 50 mM KCl, 10 mM Tris buffer, pH 8–8.4, and 100 μg/ml gelatin.

The primer is preferably single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the thermostable enzyme. The exact lengths of the primers will depend on many factors, including temperature, source of primer and use of the method. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15–25 nucleotides, although it may contain more or fewer nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with template.

The primers herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5′ end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to be amplified to hybridize therewith and thereby form a template for synthesis of the extension product of the other primer. However, for detection purposes, particularly using labeled sequence-specific probes, the primers typically have exact complementarity to obtain the best results.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "thermostable enzyme" refers to an enzyme which is stable to heat and is heat resistant and catalyzes (facilitates) combination of the nucleotides in the proper manner to form the primer extension products that are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3′ end of each primer and will proceed in the 5′ direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be a thermostable enzyme, however, which initiates synthesis at the 5′ end and proceeds in the other direction, using the same process as described above.

The thermostable enzyme herein must satisfy a single criterion to be effective for the amplication reaction, i.e., the enzyme must not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded nucleic acids. Irreversible denaturation for purposes herein refers to permanent and complete loss of enzymatic activity. The heating conditions necessary for denaturation will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the nucleic acids being denatured, but typically range from about 90 to about 105° C. for a time depending mainly on the temperature and the nucleic acid length, typically about 0.5 to four minutes. Higher temperatures may be tolerated as the buffer salt concentration and/or GC composition of the nucleic acid is increased. Preferably, the enzyme will not become irreversibly denatured at about 90–100° C.

The thermostable enzyme herein preferably has an optimum temperature at which it functions that is higher than about 40° C., which is the temperature below which hybridization of primer to template is promoted, although, depending on (1) magnesium and

salt concentrations and (2) composition and length of primer, hybridization can occur at higher temperature (e.g., 45–70° C.). The higher the temperature optimum for the enzyme, the greater the specificity and/or selectivity of the primer-directed extension process. However, enzymes that are active below 40° C., e.g., at 37° C., are also within the scope of this invention provided they are heat-stable. Preferably, the optimum temperature ranges from about 50° to 90° C., more preferably 60–80° C.

The thermostable enzyme herein may be obtained from any source and may be a native or recombinant protein. Examples of enzymes that have been reported in the literature as being resistant to heat include heat-stable polymerases, such as, e.g., polymerases extracted from the thermophilic bacteria *Thermus flavus, Thermus ruber, Thermus thnrmophilus, Bacillus stearothermophilus* (which has a somewhat lower temperature optimum than the others listed), *Thermus aquaticus, Thermus lacteus, Thermus rubens,* and *Methanothermus fervidus.*

The preferred thermostable enzyme herein is a DNA polymerase isolated from *Thermus aquaticus.* Various strains thereof are available from the American Type Culture Collection, Rockville, Md., and are described by T. D. Brock, *J. Bact.* (1969) 98:289–297, and by T. Oshima, *Arch. Microbiol.* (1978) 117: 189–196. One of these preferred strains is strain YT-1.

For recovering the native protein the cells are grown using any suitable technique. One such technique is described by Kaledin et al., *Biokhimiya* (1980), supra, the disclosure of which is incorporated herein by reference. Briefly, the cells are grown on a medium, in one liter, of nitrilotriacetic acid (100 mg), tryptone (3 g), yeast extract (3 g), succinic acid (5 g), sodium sulfite (50 mg), riboflavin (1 mg), K_2HPO_4 (522 mg), $MgSO_4$(480 mg), $CaCl_2$ (222 mg), NaCl (20 mg), and trace elements. The pH of the medium is adjusted to 8.0±0.2 with KOH. The yield is increased if cultivated with vigorous aeration up to 20 g/liter of cells at a temperature of 70° C. Cells in the late logarithmic stage (determined by absorbance at 550 nm) are collected by centrifugation, washed with a buffer and stored frozen at −20° C.

In another method for growing the cells, described in Chien et al., *J. Bacteriol.* (1976), supra, the disclosure of which is incorporated herein by reference, a defined mineral salts medium containing 0.3% glutamic acid supplemented with 0.1 mg/1 biotin, 0.1 mg/1 thiamine, and 0.05 mg/l nicotinic acid is employed. The salts include nitrilotriacetic acid, $CaSO_4$, $MgSO_4$, NaCl, KNO_3, $NaNO_3$, $ZnSO_4$, H_3BO_3, $CuSO_4$, $NaMoO_4$, $CoCl_2$, $FeCl_3$, $MnSO_4$, and Na_2HPO_4. The pH of the medium is adjusted to 8.0 with NaOH.

In the Chien et al. technique, the cells are grown initially at 75° C. in a water bath shaker. On reaching a certain density, 1 liter of these cells is transferred to 16-liter carbons which are placed in hot-air incubators. Sterile air is bubbled through the cultures and the temperature maintained at 75° C. The cells are allowed to grow for 20 hours before being collected by centrifugation.

After cell growth, the isolation and purification of the enzyme take place in six stages, each of which is carried out at a temperature below room temperature, preferably about 4° C.

In the first stage or step, the cells, if frozen, are thawed, disintegrated by ultrasound, suspended in a buffer at about pH 7.5, and centrifuged.

In the second stage, the supernatant is collected and then fractionated by adding a salt such as dry ammonium sulfate. The appropriate fraction (typically 45–75% of saturation) is collected, dissolved in a 0.2M potassium phosphate buffer preferably at pH 6.5, and dialyzed against the same buffer.

The third step removes nucleic acids and some protein. The fraction from the second stage is applied to a DEAE-cellulose column equilibrated with the same buffer as used above. Then the column is washed with the same buffer and the flow-through protein-containing fractions, determined by absorbance at 280 nm, are collected and dialyzed against a 10 mM potassium phosphate buffer, preferably with the same ingredients as the first buffer, but at a pH of 7.5.

In the fourth step, the fraction so collected is applied to a hydroxyapatite column equilibrated with the buffer used for dialysis in the third step. The column is then washed and the enzyme eluted with a linear gradient of a buffer such as 0.01M to 0.5M potassium phosphate buffer at pH 7.5 containing 10 mM 2-mercaptoethanol and 5% glycerine. The pooled fractions containing thermostable enzyme (e.g., DNA polymerase) activity are dialyzed against the same buffer used for dialysis in the third step.

In the fifth stage, the dialyzed fraction is applied to a DEAE-cellulose column, equilibrated with the buffer used for dialysis in the third step. The column is then washed and the enzyme eluted with a linear gradient of a buffer such as 0.01 to 0.6M KCl in the buffer used for dialysis in the third step. Fractions with thermostable enzyme activity are then tested for contaminating deoxyribonucleases (endo- and exonucleasesl using any suitable procedure. For example, the endonuclease activity may be determined electrophoretically from the change in molecular weight of phage λ DNA or supercoiled plasmid DNA after incubation with an excess of DNA polymerase. Similarly, exonuclease activity may be determined electrophoretically from the change in molecular weight of DNA after treatment with a restriction enzyme that cleaves at several sites.

The fractions determined to have no deoxyribonuclease activity are pooled and dialyzed against the same buffer used in the third step.

In the sixth step, the pooled fractions are placed on a phosphocellulose column with a set bed volume. The column is washed and the enzyme eluted with a linear gradient of a buffer such as 0.01 to 0.4M KCl in a potassium phosphate buffer at pH 7.5. The pooled fractions having thermostable polymerase activity and no deoxyribonuclease activity are dialyzed against a buffer at pH 8.0.

The molecular weight of the dialyzed product may be determined by any technique, for example, by SDS PAGE using protein molecular weight markers. The molecular weight of one of the preferred enzymes herein, the DNA polymerase purified from *Thermus aquaticus,* is determined by the above method to be about 86,000–90,000 daltons.

The thermostable enzyme of this invention may also be produced by recombinant DNA techniques, as the gene encoding this enzyme has been cloned from *Thermus aquaticus* genomic DNA. The complete coding sequence for the *Thermus aquaticus* (Taq) polymerase can be derived from bacteriophage CH35:Taq#4-2 on an approximately 3.5 kilobase (kb) BglII-Asp718 (partial) restriction fragment contained within an ~18 kb genomic DNA insert fragment. This bacteriophage was

deposited with the American Type Culture Collection (ATCC) on May 28, 1987 and has accession No. ATCC 40336. Alternatively, the gene can be constructed by ligating an ~750 base pair (bp) BglII-HindIII restriction fragment isolated from plasmid pFC83 (ATCC 67422 deposited May 28, 1987) to an ~2.8 kb HindIII-Asp718 restriction fragment isolated from plasmid pFC85 (ATCC 67421 deposited May 28, 1987). The pFC83 restriction fragment comprises the amino-terminus encoding region of the Taq polymerase gene while the restriction fragment from pFC85 comprises the carboxyl-terminus encoding region. Thus, ligation of these two fragments into a correspondingly digested vector with appropriate control sequences will result in the translation of a full-length Taq polymerase.

It has also been found that the entire coding sequence of the Taq polymerase gene is not required to recover a biologically active gene product with the desired enzymatic activity. Amino-terminal deletions wherein approximately one-third of the coding sequence is absent have resulted in producing a gene product that is quite active in polymerase assays.

In addition to the N-terminal deletions, individual amino acid residues in the peptide chain comprising Taq polymerase may be modified by oxidation, reduction, or other derivatization, and the protein may be cleaved to obtain fragments that retain activity. Such alterations that do not destroy activity do not remove the DNA sequence encoding such protein from the definition of gene.

Thus, modifications to the primary structure itself by deletion, addition, or alteration of the amino acids incorporated into the sequence during translation can be made without destroying the activity of the protein. Such substitutions or other alterations result in proteins having an amino acid sequence encoded by DNA falling within the contemplated scope of the present invention.

Polyclonal antiserum from rabbits immunized with the purified 86,000–90,000 dalton polymerase of this invention was used to probe a *Thermus aquaticus* partial genomic expression library to obtain the appropriate coding sequence as described below. The cloned genomic sequence can be expressed as a fusion polypeptide, expressed directly using its own control sequences, or expressed by constructions using control sequences appropriate to the particular host used for expression of the enzyme.

Of course, the availability of DNA encoding these sequences provides the opportunity to modify the codon sequence so as to generate mutein forms also having DNA polymerase activity.

Thus, these tools can provide the complete coding sequence for Taq DNA polymerase from which expression vectors applicable to a variety of host systems can be constructed and the coding sequence expressed. It is also evident from the foregoing that portions of the Taq polymerase-encoding sequence are useful as probes to retrieve other thermostable polymerase-encoding sequences in a variety of species. Accordingly, portions of the genomic DNA encoding at least six amino acids can be replicated in *E. coli* and the denatured forms used as probes or oligodeoxyribonucleotide probes can be synthesized which encode at least six amino acids and used to retrieve additional DNAs encoding a thermostable polymerase. Because there may not be a precisely exact match between the nucleotide sequence in the *Thermus aquaticus* form and that in the corresponding portion of other species, oligomers containing approximately 18 nucleotides (encoding the six amino acid stretch) are probably necessary to obtain hybridization under conditions of sufficient stringency to eliminate false positives. The sequences encoding six amino acids would supply information sufficient for such probes.

Suitable Hosts, Control Systems and Methods

In general terms, the production of a recombinant form of Taq polymerase typically involves the following:

First, a DNA is obtained that encodes the mature (used here to include all muteins) enzyme or a fusion of the Taq polymerase to an additional sequence that does not destroy its activity or to an additional sequence cleavable under controlled conditions (such as treatment with peptidase) to give an active protein. If the sequence is uninterrupted by introns it is suitable for expression in any host. This sequence should be in an excisable and recoverable form.

The excised or recovered coding sequence is then preferably placed in operable linkage with suitable control sequences in a replicable expression vector. The vector is used to transform a suitable host and the transformed host cultured under favorable conditions to effect the production of the recombinant Taq polymerase. Optionally the Taq polymerase is isolated from the medium or from the cells; recovery and purification of the protein may not be necessary in some instances, where some impurities may be tolerated.

Each of the foregoing steps can be done in a variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in appropriate hosts. The constructions for expression vectors operable in a variety of hosts are made using appropriate replicons and control sequences, as set forth below. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to provide an excisable gene to insert into these vectors.

The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene. Generally, procaryotic, yeast, insect or mammalian cells are presently useful as hosts. Procaryotic hosts are in general the most efficient and convenient for the production of recombinant proteins and therefore preferred for the expression of Taq polymerase.

In the particular case of Taq polymerase, evidence indicates that considerable deletion at the N-terminus of the protein may occur under both recombinant and native conditions, and that the activity of the protein is still retained. It appears that the native proteins isolated may be the result of proteolytic degradation, and not translation of a truncated gene. The mutein produced from the truncated gene of plasmid pFC85 is, however, fully active in assays for DNA polymerase, as is that produced from DNA encoding the full-length sequence. Since it is clear that certain N-terminal shortened forms are active, the gene constructs used for expression of the polymerase may also include the corresponding shortened forms of the coding sequence.

Control Sequences and Corresponding Hosts

Procaryotes most frequently are represented by various strains of *E. coli.* However, other microbial strains may also be used, such as bacilli, for example, *Bacillus subtilis,* various species of *Pseudomonas,* or other bacte-

rial strains. In such procaryotic systems, plasmid vectors that contain replication sites and control sequences derived from a species compatible with the host are used. For example, *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species by. Bolivar, et al., *Gene* (1977) 2:95. pBR322 contains genes for ampicillin and tetracycline resistance, and thus provides additional markers that can be either retained or destroyed in constructing the desired vector. Commonly used procaryotic control sequences, which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the β-lactamase (penicillinase) and lactose (lac) promoter systems (Chang, et al., *Nature* (1977) 198:1056), the tryptophan (trp) promoter system (Goeddel, et al., *Nucleic Acids Res.* (1980) 8:4057) and the lambda-derived P_L promoter (Shimatake, et al., *Nature* (1981) 292:128) and N-gene ribosome binding site, which has been made useful as a portable control cassette (as set forth in U.S. Pat. No. 4,711,845, filed Dec. 24, 1984), which comprises a first DNA sequence that is the P_L promoter operably linked to a second DNA sequence corresponding to N_{RBS} upstream of a third DNA sequence having at least one restriction site that permits cleavage within six bp 3′ of the N_{RBS} sequence. Also useful is the phosphatase A (phoA) system described by Chang, et al. in European Patent Publication No. 196,864 published Oct. 8, 1986, assigned to the same assignee and incorporated herein by reference. However, any available promoter system compatible with procaryotes can be used.

In addition to bacteria, eucaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of *Saccharomyces cerevisiae,* Baker's yeast, are most used, although a number of other strains are commonly available. While vectors employing the 2 micron origin of replication are illustrated (Broach, J. R., *Meth. Enz.* (1983) 101:307), other plasmid vectors suitable for yeast expression are known (see, for example, Stinchcomb, et al., *Nature* (1979) 282:39, Tschempe, et al., *Gene* (1980) 10:157 and Clarke, L., et al., *Meth. Enz.* (1983) 101:300). Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess, et al., *J. Adv. Enzyme Reg.* (1968) 7:149; Holland, et al., *Biotechnology* (1978) 17:4900).

Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman, et al., *J. Biol. Chem.* (1980) 255.2073), and those for other glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other promoters that have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose ultilization (Holland, supra).

It is also believed that terminator sequences are desirable at the 3′ end of the coding sequences. Such terminators are found in the 3′ untranslated region following the coding sequences in yeast-derived genes. Many of the vectors illustrated contain control sequences derived from the enolase gene containing plasmid peno46 (Holland, M. J., et al., *J. Biol. Chem.* (1981) 256:1385) or the LEU2 gene obtained from YEp13 (Broach, J., et al., *Gene* (1978) 8:121); however, any vector containing a yeast-compatible promoter, origin of replication, and other control sequences is suitable.

It is also, of course, possible to express genes encoding polypeptides in eucaryotic host cell cultures derived from multicellular organisms. See, for example, *Tissue Culture,* Academic Press, Cruz and Patterson, editors (1973). Useful host cell lines include murine myelomas N51, VERO and HeLa cells, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers, et al., *Nature* (1978) 273:113), or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papiloma virus, or avian sarcoma viruses, or immunoglobulin promoters and heat shock promoters. A system for expressing DNA in mammalian systems using the BPV as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. General aspects of mammalian cell host system transformations have been described by Axel, U.S. Pat. No. 4,399,216. It now appears, also, that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream of the promoter region. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes.

Plant cells are also now available as hosts, and control sequences compatible with plant cells such as the nopaline synthase promoter and polyadenylation signal sequences (Depicker, A., et al., *J. Mol. Appl. Gen.* (1982) 1:561) are available.

Recently, in addition, expression systems employing insect cells utilizing the control systems provided by baculovirus vectors have been described (Miller, D. W., et al., in *Genetic Engineering* (1986) Setlow, J. K. et al., eds., Plenum Publishing, Vol. 8, pp. 277–297). These systems are also successful in producing Taq polymerase.

Transformations

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S. N., *Proc. Natl. Acad. Sci. (USA)* (1972) 69:2110 is used for procaryotes or other cells that contain substantial cell wall barriers. Infection with *Agrobacterium tumefaciens* (Shaw, C. H., et al., *Gene* (1983) 23:315) is used for certain plant cells. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology* (1978) 52:546 is preferred. Transformations into yeast are carried out according to the method of Van Solingen, P., et al., *J. Bact.* (1977) 130:946 and Hsiao, C. L., et al., *Proc. Natl. Acad. Sci. (USA)* (1979) 76:3829.

Construction of a λgt11 Expression Library

The strategy for isolating DNA encoding desired proteins such as the Taq polymerase encoding DNA, using the bacteriophage vector lambda gt11, is as follows. A library can be constructed of EcoRI-flanked AluI fragments, generated by complete digestion of *Thermus aquaticus* DNA, inserted at the *EcoRI* site in

the lambda gt11 phage (Young and Davis, *Proc. Natl. Acad. Sci USA* (1983) 80:1194–1198). Because the unique EcoRI site in this bacteriophage is located in the carboxyl-terminus of the β-galactosidase gene, inserted DNA (in the appropriate frame and orientation) is expressed as protein fused with β-galactosidase under the control of the lactose operon prompter/operator.

Genomic expression libraries are then screened using the antibody plaque hybridization procedure. A modification of this procedure, referred to as "epitope selection," uses antiserum against the fusion protein sequence encoded by the phage, to confirm the identification of hybridized plaques. Thus, this library of recombinant phages could be screened with antibodies that recognize the 86,000–90,000 dalton Taq polymerase in order to identify phage that carry DNA segments encoding the antigenic determinants of this protein.

Approximately 2×10^5 recombinant phage are screened using total rabbit Taq polymerase antiserum. In this primary. screen, positive signals are detected and one or more of these plaques are purified from candidate plaques which failed to react with preimmune serum and reacted with immune serum and analyzed in some detail. To examine the fusion proteins produced by the recombinant phage, lysogens of the phage in the host Y1089 are produced. Upon induction of the lysogens and gel electrophoresis of the resulting proteins, each lysogen may be observed to produce a new protein, not found in the other lysogens, or duplicate sequences may result. Phage containing positive signals are picked., in this case, one positive plaque was picked for further identification and replated at lower densities to purify recombinants and the purified clones were analyzed by size class via digestion with EcoRI restriction enzyme. Probes can then be made of the isolated DNA insert sequences and labeled appropriately and these probes can be used in conventional colony or plaque hybridization assays described in Maniatis et al., *Molecular Cloning: A Laboratory Manual* (1982), the disclosure of which is incorporated herein by reference.

The labeled probe was used to probe a second genomic library constructed in a Charon 35 bacteriophage (Wilhelmine, A. M. et al., *Gene* (1983) 26:171–179). This library was made from Sau3A partial digestions of genomic *Thermus aquaticus* DNA and size fractionated fragments (15-20 kb) were cloned into the BamHI site of the Charon 35 phage. The probe was used to isolate phage containing DNA encoding the Taq polymerase. One of the resulting phage, designated CH35:Taq#4-2, was found to contain the entire gene sequence. Partial sequences encoding portions of the gene were also isolated.

Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques that are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions that are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 μg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 μl of buffer solution; in the examples herein, typically an excess of restriction enzyme is used to ensure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37° C. are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/-chloroform, and may be followed by o ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in *Methods in Enzymology* (1980) 65:499–560.

Restriction-cleaved fragments may be blunt-ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 minutes at 20 to 25° C. in 50 mM Tris pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT and 50–100 μM dNTPs. The Klenow fragment fills in at 5′ sticky ends, but chews back protruding 3′ single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with phenol/-chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

Synthetic oligonucleotides may be prepared using the triester method of Matteucci, et al., (*J. Am. Chem. Soc.* (1981) 103:3185–3191) or using automated synthesis methods. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nM substrate in the presence of 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1–2 mM ATP. If kinasing is for labeling of probe, the ATP will contain high specific activity γ-^{32}P.

Ligations are performed in 15–30 μl volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 μg/ml BSA, 10 mM-50 mM NaCl, and either 40 μM ATP, 0.01–0.02 (Weiss) units T4 DNA ligase at 0° C. (for "sticky end" ligation) or 1 mM ATP, 0.3–0.6 (Weiss) units T4 DNA ligase at 14° C. (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33–100 μg/ml total DNA concentrations (5–100 nM total end concentration). Intermolecular blunt end ligations (usually employing a 10–30 fold molar excess of linkers) are performed at 1 μM total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5′ phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{+2} using about 1 unit of BAP per mg of vector at 60° C. for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors that have been double digested by additional restriction enzyme digestion of the unwanted fragments.

Modification of DNA Sequences

For portions of vectors derived from cDNA or genomic DNA that require sequence modifications, site-specific primer-directed mutagenesis is used. This technique is now standard in the art, and is conducted using a primer synthetic oligonucleotide complementary to a single-stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells that harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The plaques are transferred to nitrocellulose filters and the "lifts" hybridized with kinased synthetic primer at a temperature that permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hydbrodization. Plaques that hybridize with the probe are then picked and cultured, and the DNA is recovered.

Verification of Construction

In the constructions set forth below, correct ligations for plasmid construction are confirmed by first transforming *E. coli* strain MM294, or other suitable host, with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers, depending on the mode of plasmid construction, as is understood in the ar. Plasmids from the transformants are then prepared according to the method of Clewell, D. B., et al., *Proc. Natl. Acad. Sci. (USA)* (1969) 62:1159, optionally following chloramphenicol amplication (Clewell, D. B., *J. Bacteriol.* (1972). 110:667) 110:667). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy method of Sanger, F., et al., *Proc. Natl. Acad. Sci. (USA)* (1977) 74:5463 as further described by Messing, et al., *Nucleic Acids Res.* (1981) 9:309, or by the method of Maxam, et al., *Methods in Enzymology* (1980)65:499.

Host Exemplified

Host strains used in cloning and expression herein are as follows:

For cloning and sequencing, and for expression of constructions under control of most bacterial promoters, *E. coli* strain MM294 obtained from *E. coli* Genetic Stock Center GCSC #6135, was used as the host. For expression under control of the P_LN_{RBS} promoter, *E. coli* strain K12 MC1000 lambda lysogen, $N_7N_{53}cI857$ $SusP_{80}$, ATCC 39531 may be used. Used herein is *E. coli* DG116, which was deposited with ATCC (ATCC 53606) on Apr. 7, 1987.

For M13 phage recombinants, *E. coli* strains susceptible to phage infection, such as *E. coli* K12 strain DG98, are employed. The DG98 strain has been deposited with ATCC July 13, 1984 and has accession number 39768.

Mammalian expression can be accomplished in COS-7 COS-A2, CV-1, and murine cells, and insect cell based expression in *Spodoptera frugipeida*).

Stabilization of Enzyme Activity

For long-term stability, the enzyme herein must be stored in a buffer that contains one or more non-ionic polymeric detergents. Such detergents are generally those that have a molecular weight in the range of approximately 100 to 250,000, preferably about 4,000 to 200,000 daltons and stabilize the enzyme at a pH of from about 3.5 to about 9.5, preferably from about 4 to 8.5. Examples of such detergents include those specified on pages 295–298 of McCutcheon's *Emulsifiers & Detergents,* North American edition (1983), published by the McCutcheon Division of MC Publishing Co., 175 Rock Road, Glen Rock, N.J. (USA), the entire disclosure of which is incorporated herein by reference. Preferably, the detergents are selected from the group comprising ethoxylated fatty alcohol ethers and lauryl ethers, ethoxylated alkyl phenols, octylphenoxy polyethoxy ethanol compounds, modified oxyethylated and/or oxypropylated straight-chain alcohols, polyethylene glycol monooleate compounds, polysorbate compounds, and phenolic fatty. alcohol ethers. More particularly preferred are Tween 20, from ICI Americas Inc., Wilmington, Del., which is a polyoxyethylated (20) sorbitan monolaurate, and Iconol TM NP-40, from BASF Wyandotte Corp. Parsippany, N.J., which is an ethoxylated alkyl Phenol (nonyl).

The thermostable enzyme of this invention may be used for any purpose in which such enzyme is necessary or desirable. In a particularly preferred embodiment, the enzyme herein is employed in the amplification protocol set forth below.

Amplification Protocol

The amplification protocol using the enzyme of the present invention may be the process for amplifying existing nucleic acid sequences that is disclosed and claimed in copending U.S. Pat. No. 4,683,202 filed Oct. 25, 1985, the disclosure of which is incorporated herein by reference. Preferably, however, the enzyme herein is used in the amplification process disclosed and claimed in copending U.S. patent application Ser. No. 899,513 filed Aug. 22, 1986, wherein Cetus Corporation is the assignee, as in the present invention, entitled "Process for Amplifying, Detecting, and/or Cloning Nucleic Acid Sequences Using A Thermostable Enzyme". The disclosure of said latter application is herein incorporated by reference.

More specifically, the amplification method of the latter application involves amplifying at least one specific nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, wherein if the nucleic acid is double-stranded, it consists of two separated complementary strands of equal or unequal length, which process comprises:

(a) contacting each nucleic acid strand with four different nucleotide triphosphates and one oligonucleotide primer for each different specific sequence being amplified, wherein each primer is selected to be substantially complementary. to different strands of each specific sequence, such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, said contacting being at a temperature which promotes hybridization of each primer to its complementary nucleic acid strand;

(b) contacting each nucleic acid strand, at the same time as or after step (a), with a DNA polymerase from

Thermus aquaticus which enables combination of the nucleotide triphosphates to form primer extension products complementary to each strand of each nucleic acid;

(c) maintaining the mixture from step (b) at an effective temperature for an effective time to promote the activity of the enzyme, and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each nucleic acid strand template, but not so high as to separate each extension product from its complementary strand template;

(d) heating the mixture from step (c) for an effective time and at an effective temperature to separate the primer extension products from the templates on which they were synthesized to produce single-stranded molecules, but not so high as to denature irreversibly the enzyme;

(e) cooling the mixture from step (d) for an effective time and to an effective temperature to promote hybridization of each primer to each of the single-stranded molecules produced in step (d); and

(f) maintaining the mixture from step (e) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each nucleic acid strand template produced in step (d), but not so high as to separate each extension product from its complementary strand template wherein the effective time and temperatures in steps (e) and (f) may coincide (steps (e) and (f) are carried out simultaneously), or may be separate.

Steps (d)–(f) may be repeated until the desired level of sequence amplification is obtained.

The amplification method is useful not only for producing large amounts of an existing completely specified nucleic acid sequence, but also for producing nucleic acid sequences which are known to exist but are not completely specified. In either case an initial copy of the sequence to be amplified must be available, although it need not be pure or a discrete molecule.

In general, the amplification process involves a chain reaction for producing, in exponential quantities relative to the number of reaction steps involved, at least one specific nucleic acid sequence given (a) that the ends of the required sequence are known in sufficient detail that oligonucleotides can be synthesized which will hybridize to them, and {b) that a small amount of the sequence is available to initiate the chain reaction. The product of the chain reaction will be a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

Any nucleic acid sequence, in purified or nonpurified form, can be utilized as the starting nucleic acid(s), provided it contains or is suspected to contain the specific nucleic acid sequence desired. Thus, the process may employ, for example, DNA or RNA, including messenger RNA, which DNA or RNA may be single-stranded or double-stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any of these nucleic acids may also be employed, or the nucleic acids produced from a previous amplification reaction herein using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified may be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid.

It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as a portion of the β-globin gene contained in whole human DNA (as exemplified in Saiki et al., *Science,* 230, 1530–1534 (1985)) or a portion of a nucleic acid sequence due to a particular microorganism which organism might constitute only a very minor fraction of a particular biological sample. The starting nucleic acid sequence may contain more than one desired specific nucleic acid sequence which may be the same or different. Therefore, the amplification process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules.

The nucleic acid(s) may be obtained from any source, for example, from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, organelles, and higher organisms such as plants or animals. DNA or RNA may be extracted from blood, tissue material such as chorionic villi, or amniotic cells by a variety of techniques such as that described by Maniatis et al., supra, p. 280–281.

If probes are used which are specific to a sequence being amplified and thereafter detected, the cells may be directly used without extraction of the nucleic acid if they are suspended in hypotonic buffer and heated to about 90–100° C., until cell lysis and dispersion of intracellular components occur, generally 1 to 15 minutes. After the heating step the amplification reagents may be added directly to the lysed cells.

Any specific nucleic acid sequence can be produced by the amplification process. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail so that two oligonucleotide primers can be prepared which will hybridize to different strands of the desired sequence and at relative positions along the sequence such that an extension product synthesized from one primer, when it is separated from its template (complement), can serve as a template for extension of the other primer into a nucleic acid sequence of defined length. The greater the knowledge about the bases at both ends of the sequence, the greater can be the specificity of the primers for the target nucleic acid sequence, and thus the greater the efficiency of the process.

It will be understood that the word "primer" as used hereinafter may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding the terminal sequence(s) of the fragment to be amplified. For instance, in the case where a nucleic acid sequence is inferred from protein sequence information, a collection of primers containing sequences representing all possible codon variations based on degeneracy of the genetic code will be used for each strand. One primer from this collection will be homologous with the end of the desired sequence to be amplified.

The oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods described above, or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et al., *Tetrahedron Letters* (1981), 22:1859–1862. One method for synthesizing oligonucle-

otides on a modified solid support is described in U.S. Pat. No. 4,458,066. It is also possible to use a primer which has been isolated from a biological source (such as a restriction endonuclease digest).

The specific nucleic acid sequence is produced by using the nucleic acid containing that sequence as a template. The first step involves contacting each nucleic acid strand with four different nucleotide triphosphates and one oligonucleotide primer for each different nucleic acid sequence being amplified or detected. If the nucleic acids to be amplified or detected are DNA, then the nucleotide triphosphates are dATP, dCTP, dGTP and TTP.

The nucleic acid strands are used as a template for the synthesis of additional nucleic acid strands. This synthesis can be performed using any suitable method. Generally it occurs in a buffered aqueous solution, preferably at a pH of 7–9, most preferably about 8. Preferably, a molar excess (for cloned nucleic acid, usually about 1000:1 primer:template, and for genomic nucleic acid, usually about 10^6:1 primer:template) of the two oligonucleotide primers is added to the buffer containing the separated template strands. It is understood, however, that the amount of complementary strand may not be known if the process herein is used for diagnostic applications, so that the amount of primer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, however, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process.

Preferably the concentration of nucleotide triphosphates is 150–200 μM each in the buffer for amplification and $MgCl_2$ is present in the buffer in an amount of 1.5–2 mM to increase the efficiency and specificity of the reaction.

The resulting solution is then treated according to whether the nucleic acids being amplified or detected are double or single-stranded. If the nucleic acids are single-stranded, then no denaturation step need by employed, and the reaction mixture is held at a temperature which promotes hybridization of the primer to its complementary target (template) sequence. Such temperature is generally from about 35° C. to 65° C. or more, preferably about 37–60° C. for an effective time, generally one-half to five minutes, preferably one-three minutes. Preferably, 45–58° C. is used for Taq polymerase and >15-mer primers to increase the specificity of primer hybridization. Shorter primers need lower temperatures.

The complement to the original single-stranded nucleic acid may be synthesized by adding one or two oligonucleotide primers thereto. If an appropriate single primer is added, a primer extension product is synthesized in the presence of the primer, the DNA polymerase from *Thermus aquaticus* and the nucleotide triphosphates. The product will be partially complementary to the single-stranded nucleic acid and will hybridize with the nucleic acid strand to form a duplex of strands of unequal length which may then be separated into single strands as described above to produce two single separated complementary strands. Alternatively, two appropriate primers may be added to the single-stranded nucleic acid and the reaction carried out.

If the nucleic acid contains two strands, it is necessary to separate the strands of the nucleic acid before it can be used as the template. This strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One preferred physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 90° to 105° C. for times generally ranging from about 0.5 to 5 minutes. Preferably the effective denaturing temperature is 90–100° C. for 0.5 to 3 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and in the presence of riboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are describe by Kuhn Hoffmann-Berling, *CSH-Quantitative Biology,* 43:63 (1978), and techniques for using RecA are reviewed in C. Radding, *Ann. Rev. Genetics,* 16:405–37 (1982). The denaturation produces two separated complementary strands of equal or unequal length.

If the double-stranded nucleic acid is denatured by heat, the reaction mixture is allowed to cool to a temperature which promotes hybridization of each primer present to its complementary. target (template) sequence. This temperature is usually from about 35° C. to 65° C. or more, depending on reagents, preferably 37–60° C., maintained for an effective time, generally 0.5 to 5 minutes, and preferably 1–3 minutes. In practical terms, the temperature is simply lowered from about 95° C. to as low as 37° C., preferably to about 45–58° C. for Taq polymerase, and hybridization occurs at a temperature within this range.

Whether the nucleic acid is single- or double-stranded, the DNA polymerase from *Thermus aquaticus* may be added at the denaturation step or when the temperature is being reduced to or is in the range for promoting hybridization. The reaction mixture is then heated to a temperature at which the activity of the enzyme is promoted or optimized, i.e., a temperature sufficient to increase the activity of the enzyme in facilitating synthesis of the primer extension products from the hybridized primer and template. The temperature must actually be sufficient to synthesize an extension product of each primer which is complementary to each nucleic acid template, but must not be so high as to denature each extension product from its complementary template (i.e., the temperature is generally less than about 80° C.–90° C.).

Depending mainly on the types of enzyme and nucleic acid(s) employed, the typical temperature effective for this synthesis reaction generally ranges from about 40° to 80° C., preferably 50–75° C. The temperature more preferably ranges from about 65–75° C. when a DNA polymerase from *Thermus aquaticus* is employed. The period of time required for this synthesis may range from about 0.5 to 40 minutes or more, depending mainly on the temperature, the length of the nucleic acid, the enzyme and the complexity of the nucleic acid mixture, preferably one to three minutes. If the nucleic acid is longer, a longer time period is generally required. The presence of dimethylsulfoxide (DMSO) is not necessary or recommended because DMSO was found to inhibit Taq polymerase enzyme activity.

The newly synthesized strand and its complementary nucleic acid strand form a double-stranded molecule which is used in the succeeding steps of the process. In the next step, the strands of the double-stranded molecule are separated by heat denaturation at a temperature effective to denature the molecule, but not so high that the thermostable enzyme is completely and irreversibly denatured or inactivated. Depending mainly on the type of enzyme and the length of nucleic acid, this temperature generally ranges from about 90° to 105° C., more preferably 90–100° C., and the time for denaturation typically ranges from 0.5 to four minutes, depending mainly on the temperature and nucleic acid length.

After this time, the temperature is decreased to a level which promotes hybridization of the primer to its complementary single-stranded molecule (template) produced from the previous step. Such temperature is described above.

After this hybridization step, or in lieu of (or concurrently with) the hybridization step, the temperature is adjusted to a temperature that is effective to promote the activity of the thermostable enzyme to enable synthesis of a primer extension product using as template the newly synthesized strand from the previous step. The temperature again must not be so high as to separate (denature) the extension product from its template, as previously described (usually from 40° to 80° C. for 0.5 to 40 minutes, preferably 50° to 70° C. for one-three minutes). Hybridization may occur during this step, so that the previous step of cooling after denaturation is not required. In such a case, using simultaneous steps, the preferred temperature range is 50–70° C.

The heating and cooling steps of strand separation, hybridization, and extension product synthesis can be repeated as often as needed to produce the desired quantity of the specific nucleic acid sequence, depending on the ultimate use. The only limitation is the amount of the primers, thermostable enzyme and nucleotide triphosphates present. Preferably, the steps are repeated at least twice. For use in detection, the number of cycles will depend, e.g., on the nature of the sample. For example, fewer cycles will be required if the sample being amplified is pure. If the sample is a complex mixture of nucleic acids, more cycles will be required to amplify the signal sufficiently for its detection. For general amplification and detection, preferably the process is repeated at least 20 times.

When labeled sequence-specific probes are employed as described below, preferably the steps are repeated at least five times. When human genomic DNA is employed with such probes, the process is repeated preferably 15–30 times to amplify the sequence sufficiently that a clearly detectable signal is produced, i.e., so that background noise does not interfere with detection.

As will be described in further detail below, the amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion.

No additional nucleotides, primers, or thermostable enzyme need be added after the initial addition, provided that the enzyme has not become denatured or inactivated irreversibly, in which case it is necessary to replenish the enzyme after each denaturing step. Addition of such materials at each step, however, will not adversely affect the reaction.

When it is desired to produce more than one specific nucleic acid sequence from the first nucleic acid or mixture of nucleic acids, the appropriate number of different oligonucleotide primers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers are utilized. Two of the primers are specific for one of the specific nucleic acid sequences and the other two primers are specific for the second specific nucleic acid sequence. In this manner, each of the two different specific sequences can be produced exponentially by the present process.

After the appropriate length of time has passed to produce the desired amount of the specific nucleic acid sequence, the reaction may be halted by inactivating the enzyme in any known manner (e.g., by adding EDTA, phenol, SDS or $CHCl_3$) or by separating the components of the reaction.

The amplification process may be conducted continuously. In one embodiment of an automated process, the reaction mixture may be temperature cycled such that the temperature is programmed to be controlled at a certain level for a certain time.

One such instrument for this purpose is the automated machine for handling the amplification reaction of this invention described in copending U.S. Ser. No. 833,368 filed Feb. 25, 1986 entitled "Apparatus And Method For Performing Automated Amplification of Nucleic Acid Sequences And Assays Using Heating And Cooling Steps," the disclosure of which is incorporated herein by reference. Briefly, this instrument utilizes a liquid handling system under computer control to make liquid transfers of enzyme stored at a controlled temperature in a first receptacle into a second receptacle whose temperature is controlled by the computer to conform to a certain incubation profile. The second receptacle stores the nucleic acid sequence(s) to be amplified plus the nucleotide triphosphates and primers. The computer includes a user interface through which a user can enter process parameters that control the characteristics of the various steps in the amplification sequence such as the times and temperatures of incubation, the amount of enzyme to transfer, etc.

A preferred machine that may be employed utilizes temperature cycling without a liquid handling system because the enzyme need not be transferred at every cycle. Such a machine is described more completely in copending U.S. application Ser. No. 899,061, filed Aug. 22, 1986, entitled "Apparatus and Method for Performing Automated Amplification of Nucleic Acid Sequences and Assays Using Heating and Cooling Steps," the disclosure of which is incorporated herein by reference. Briefly, this instrument consists of the following systems:

1. A heat-conducting container for holding a given number of tubes, preferably 500 μl tubes, which contain the reaction mixture of nucleotide triphosphates, primers, nucleic acid sequences, and enzyme.

2. A means to heat, cool, and maintain the heat-conducting container above and below room temperature, which means has an input for receiving a control signal for controlling which of the temperatures at or to which the container is heated, cooled or maintained. (These may be Peltier heat pumps available from Materials Electronics Products Corporation in Trenton, N.J. or a water heat exchanger.)

3. A computer means (e.g., a microprocessor controller), coupled to the input of said means, to generate the signals that control automatically the amplification sequence, the temperature levels, and the temperature ramping and timing.

The amplification protocol is demonstrated diagrammatically below, where double-stranded DNA contain-

ing the desired sequence [S] comprised of complementary strands [S+] and [S−] is utilized as the nucleic acid. During the first and each subsequent reaction cycle, extension of each oligonucleotide primer on the original template will produce one new ssDNA molecule product of indefinite length that terminates with only one of the primers. These products, hereafter referred to as "long products," will accumulate in a linear fashion; that is, the amount present after any number of cycles will be proportional to the number of cycles.

The long products thus produced will act as templates for one or the other of the oligonucleotide primers during subsequent cycles and will produce molecules of the desired sequence [S+] or [S−]. These molecules will also function as templates for one or the other of the oligonucleotide primers, producing further [S+] and [S−], and thus a chain reaction can be sustained that will result in the accumulation of [S] at an exponential rate relative to the number of cycles.

By products formed by oligonucleotide hybridizations other than those intended are not sel acatalytic (except in rare instancesl and thus accumulate at a linear rate.

The specific sequence to be amplified, [S], can be depicted diagrammatically as:

[S+] 5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCC 3′
[S−] 3′ TTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′

The appropriate oligonucleotide primers would be:

Primer 1: 3′GGGGGGGGGG 5′

Primer 2: 5′AAAAAAAAAA 3′

so that if DNA containing [S]

. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .

is separated into single strands and its single strands are hybridized to Primers 1 and 2, the following extension reactions can be catalyzed by a thermostable polymerase in the presence of the four nucleotide triphosphates:

3′ 5′
extends ⟵ GGGGGGGGGG Primer 1

. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
original template strand+

original template strand−
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .

Primer 2 AAAAAAAAAA ⟶ extends
5′ 3′

On denaturation of the two duplexes formed, the products are:

3′ 5′
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG
newly synthesized long product 1

5′ 3′
. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
original template strand+

3′ 5′
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .
original template strand−

5′ 3′
AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
newly synthesized ling product 2

If these four strands are allowed to rehybrimize with Primers 1 and 2 in the next cycle, the thermostable polymerase will catalyze the following reactions:

Primer 2 5′ AAAAAAAAAA ⟶ extends to here

3′. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′
newly sunthesized long product 1

extends ⟵ GGGGGGGGGG 5′ Primer 1

5′. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3′
original template strand+

Primer 2 5′ AAAAAAAAAA ⟶ extends

-continued

3'. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 5'
original template strand−

extends to here ←GGGGGGGGGG 5' Primer 1

5' AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3'
newly synthesized long product 2

If the strands of the above four duplexes are separated, the following strands are found:

5' AAAAAAAAAAXXXXXXXXXXCCCCCCCCCC 3'
newly synthesized [S+]

3'. . . zzzzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5'
first cycle synthesized long product 1

3'. . . zzzzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5'
newly synthesized long product 1

5'. . . zzzzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzz . . . 3'
original template strand+

5' AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3'
newly synthesized long product 2

3'. . . zzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . . 5'
original template strand−

3' TTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5'
newly synthesized [S−]

5' AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3'
first cycle synthesized long product 2

It is seen that each strand which terminates with the oligonucleotide sequence of one primer and the complementary sequence of the other is the specific nucleic acid sequence [S] that is desired to be produced.

The amount of original nucleic acid remains constant in the entire process, because it is not replicated. The amount of the long products increases linearly because they are produced only from the original nucleic acid. The amount of the specific sequence increases exponentially. Thus, the specific sequence will become the predominant species This is illustrated in the following table, which indicates the relative amounts of the species theoretically present after n cycles, assuming 100% efficiency at each cycle:

	Number of Double Strands After 0 to n Cycles		
Cycle Number	Template	Long Products	Specific Sequence [S]
0	1	—	—
1	1	1	0
2	1	2	1
3	1	3	4
5	1	5	26
10	1	10	1013
15	1	15	32,752
20	1	20	1,048,555
n	1	n	(2^n-n-1)

When a single-stranded nucleic acid is utilized as the template, only one long product is formed per cycle.

A sequence within a given sequence can be amplified after a given number of amplifications to obtain greater specificity of the reaction by adding after at least one cycle of amplification a set of primers that are complementary to internal sequences (that are not on o the ends) of the sequence to be amplified. Such primers may be added at any stage and will provide a shorter amplified fragment. Alternatively, a longer fragment can be prepared by using primers with non-complementary ends but having some overlap with the primers previously utilized in the amplification.

The amplification method may be utilized to clone a particular nucleic acid sequence for insertion into a suitable expression vector. The vector may be used to transform an appropriate host organism to produce the gene product of the sequence by standard methods of recombinant DNA technology. Such cloning may involve direct ligation into a vector using blunt-end ligation, or use of restriction enzymes to cleave at sites contained within the primers.

In addition, the amplification process can be used for in vitro mutagenesis. The oligodeoxyribonucleotide primers need not be exactly complementary to the DNA sequence that is being amplified. It is only necessary that they be able to hybridize to the sequence sufficiently well to be extended by the thermostable enzyme. The product of an amplification reaction wherein the primers employed are not exactly complementary to the original template will contain the sequence of the primer rather than the template, thereby introducing an in vitro mutation. In further cycles this mutation will be amplified with an undiminished efficiency because no further mispaired priming is required. The mutant thus produced may be inserted into an appropriate vector by standard molecular biological techniques and might confer mutant properties on this vector such as the potential for production of an altered protein.

The process of making an altered DNA sequence as described above could be repeated on the altered DNA using different primers to induce further sequence changes. In this way, a series of mutated sequences could gradually be produced wherein each new addition to the series could differ from the last in a minor way, but from the original DNA source sequence in an

increasingly major way. In this manner, changes could be made ultimately which were not feasible in a single step due to the inability of a very seriously mismatched primer to function.

In addition, the primer can contain as part of its sequence a non-complementary sequence, provided that a sufficient amount of the primer contains a sequence that is complementary to the strand to be amplified. For example, a nucleotide sequence that is not complementary to the template sequence (such as, e.g., a promoter, linker, coding sequence, etc.) may be attached at the 5' end of one or both of the primers, and thereby appended to the product of the amplification process. After the extension primer is added, sufficient cycles are run to achieve the desired amount of new template containing the non-complementary nucleotide insert. This allows production of large quantities of the combined fragments in a relatively short period of time (e.g., two hours or less) using a simple technique.

The amplification method may also be used to enable detection and/or characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders or cellular disorders such as cancer, e.g., oncogenes. Amplification is useful when the amount of nucleic acid available for analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells. Amplification is particularly useful if such an analysis is to be done on a small sample using nonradioactive detection techniques which may be inherently insensitive, or where radioactive techniques are being employed, but where rapid detection is desirable.

For the purposes of this discussion, genetic diseases may include specific deletions and/or mutations in genomic DNA from any organism, such as, e.g., sickle cell anemia, cystic fibrosis, α-thalassemia, β-thalassemia, and the like. Sickle cell anemia can be readily detected via oligomer restriction analysis as described by EP Patent Publication 164,054 published December 11, 1985, or via a RFLP-like analysis following amplification of the appropriate DNA sequence by the amplification method. α-Thalassemia can be detected by the absence of a sequence, and β-thalassemia can be detected by the presence of a polymorphic restriction site closely linked to a mutation that causes the disease.

All of these genetic diseases may be detected by amplifying the appropriate sequence and analyzing it by Southern blots without using radioactive probes. In such a process, for example, a small sample of DNA from, e.g., amniotic fluid containing a very low level of the desired sequence is amplified, cut with a restriction enzyme, and analyzed via a Southern blotting technique. The use of nonradioactive probes is facilitated by the high level of the amplified signal.

In another embodiment, a small sample of DNA may be amplified to a convenient level and then a further cycle of extension reactions performed wherein nucleotide derivatives which are readily detectable (such as ^{32}P-labeled or biotin-labeled nucleotide triphosphates) are incorporated directly into the final DNA product, which may be analyzed by restriction and electrophoretic separation or any other appropriate method.

In a further embodiment, the nucleic acid may be exposed to a particular restriction endonuclease prior to amplification. Since a sequence which has been cut cannot be amplified, the appearance of an amplified fragment, despite prior restriction of the DNA sample, implies the absence of a site for the endonuclease within the amplified sequence. The presence or absence of an amplified sequence can be detected by an appropriate method.

A practical application of the amplification technique, that is, in facilitating the detection of sickle cell anemia via the oligomer restriction technique [described in EP 164,054, supra, and by Saiki et al., *Bio/-Technology,* Vol 3, pp. 1008–1012 (1985)] is described in detail in the Saiki et al. *Science* article cited above. In that *Science* article, a specific amplification protocol is exemplified using a β-globin gene segment.

The amplification method herein may also be used to detect directly single-nucleotide variations in nucleic acid sequence (such as genomic DNA) using sequence-specific oligonucleotides, as described more fully in now abandoned U.S. Ser. No. 839,331 filed Mar. 13, 1986 and in copending U.S. Ser. No. 899,344 filed Aug. 22, 1986, entitled "Process for Detecting Specific Nucleotide Variations and Genetic Polymorphisms Present in Nucleic Acids," which is a continuation-in-part of U.S. Serial No. 839,331, the disclosures of both of which are incorporated herein by reference.

Briefly, in this process, the amplified sample is spotted directly on a series of membranes, and each membrane is hybridized with a different labeled sequence-specific oligonucleotide probe. After hybridization the sample is washed and the label is detected. This technique is especially useful in detecting DNA polymorphisms.

Various infectious diseases can be diagnosed by the presence in clinical samples of specific DNA sequences characteristic of the causative microorganism. These include bacteria, such as Salmonella, Chlamydia, Neisseria; viruses, such as the hepatitis viruses, and Parasites, such as the Plasmodium responsible for malaria. U.S. Patent Reexamination Certificate B1 4,358,535 issued to Falkow et al. on May 13, 1986 describes the use of specific DNA hybridization probes for the diagnosis of infectious diseases. A relatively small number of pathogenic organisms may be present in a clinical sample from an infected patient and the DNA extracted from these may constitute only a very. small fraction of the total DNA in the sample. Specific amplification of suspected pathogen-specific sequences prior to immobilization and detection by hybridization of the DNA samples could greatly improve the sensitivity and specificity of traditional procedures.

Routine clinical use of DNA probes for the diagnosis of infectious diseases would be simplified considerably if nonradioactively labeled probes could be employed as described in EP 63,879 to Ward. In this procedure biotin-containing DNA probes are detected by chromogenic enzymes linked to avidin or biotin-specific antibodies. This type of detection is convenient, but relatively insensitive. The combination of specific DNA amplification by the present method and the use of stably labeled probes could provide the convenience and sensitivity required to make the Falkow et al. and Ward procedures useful in a routine clinical setting.

A specific use of the amplification technology for detecting or monitoring for the AIDS virus is described in now abandoned copending U.S. application Ser. No. 818,127, filed Jan. 10, 1986, the disclosure of which is incorporated herein by reference and repeated in co-pending U.S. Ser. No. 935,581, filed Nov. 26, 1986, a continuation-in-part of U.S. Ser. No. 818,127. Briefly, the amplification and detection process is used with primers and probes which are designed to amplify and

detect, respectively, nucleic acid sequences that are substantially conserved among the nucleic acids in AIDS viruses and specific to the nucleic acids in AIDS viruses. Thus, the sequence to be detected must be sufficiently complementary to the nucleic acids in AIDS viruses to initiate polymerization preferably at room temperature in the presence of the enzyme and nucleotide triphosphates.

The amplification process can also be utilized to produce sufficient quantities of DNA from a single copy human gene such that detection by a simple non-specific DNA stain such as ethidium bromide can be employed to diagnose DNA directly.

In addition to detecting infectious diseases and pathological abnormalities in the genome of organisms, the amplification process can also be used to detect DNA polymorphisms which may not be associated with any pathological state.

In summary, the amplification process is seen to provide a process for amplifying one or more specific nucleic acid sequences using a chain reaction and a thermostable enzyme, in which reaction primer extension products are produced which can subsequently act as templates for further primer extension reactions. The process is especially useful in detecting nucleic acid sequences which are initially present in only very small amounts.

The following examples are offered by way of illustration only and are by no means intended to limit the scope of the claimed invention. In these examples, all percentages are by weight if for solids and by volume if for liquids, unless otherwise noted, and all temperatures are given in degrees Celsius.

EXAMPLE I

1. Synthesis of the Primers

The following two oligonucleotide primers were prepared by the method described below:

5'-ACACAACTGTGTTCACTAGC-3' (PC03)

5'-CAACTTCATCCACGTTCACC-3' (PC04)

These primers, both 20-mers, anneal to opposite strands of the genomic DNA with their 5' ends separated by a distance of 110 base pairs.

A. Automated Synthesis Procedures: The diethylphosphoramidites, synthesized according to Beaucage and Caruthers (Tetrahedron Letters (1981) 22:1859–1862) were sequentially condensed to a nucleoside derivatized controlled pore glass support using a Biosearch SAM-1. The procedure included detritylation with trichloroacetic acid in dichloromethane, condensation using benzotriazole as activating proton donor, and capping with acetic anhydride and dimethylaminopyridine in tetrahydrofuran and pyridine. Cycle time was approximately 30 minutes. Yields at each step were essentially quantitative and were determined by collection and spectroscopic examination of the dimethoxytrityl alcohol released durng detritylation.

B. Oligodeoxyribonucleotide Deprotection and Purification Procedures: The solid support was removed from the column and exposed to 1 ml concentrated ammonium hydroxide at room temperature for four hours in a closed tube. The support was then removed by filtration and the solution containing the partially protected oligodeoxynucleotide was brought to 55° C. for five hours. Ammonia was removed and the residue was applied to a preparative polyacrylamide gel. Electrophoresis was carried out at 30 volts/cm for 90 minutes after which the band containing the product was identified by UV shadowing of a fluorescent plate. The band was excised and eluted with 1 ml distilled water overnight at 4° C. This solution was applied to an Altech RP18 column and eluted with a 7–13% gradient of acetonitrile in 1% ammonium acetate buffer at pH 6.0. The elution was monitored by UV absorbance at 260 nm and the appropriate fraction collected, quantitated by UV absorbance in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge.

C. Characterization of Oligodeoxyribonucleotides: Test aliquots of the purified oligonucleotides were ^{32}P labeled with polynucleotide kinase and γ-^{32}P-ATP. The labeled compounds were examined by autoradiography of 14–20% polyacrylamide gels after electrophoresis for 45 minutes at 50 volts/cm. This procedure verifies the molecular weight. Base composition was determined by digestion of the oligodeoxyribonucleotide to nucleosides by use of venom diesterase and bacterial alkaline phosphatase and subsequent separation and quantitation of the derived nucleosides using a reverse phase HPLC column and a 10% acetonitrile, 1% ammonium acetate mobile phase.

II. Isolation of Human Genomic DNA from Cell Line

High molecular weight genomic DNA was isolated from a T cell line, Molt 4, homozygous for normal β-globin available from the Human Genetic Mutant Cell Depository, Camden, NJ as GM2219C using essentially the method of Maniatis et al., supra, p. 280–281.

III. Purification of a Polymerase From *Thermus aquaticus*

Thermus aquaticus strain YT1, available without restriction from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, as ATCC No. 25,104 was grown in flasks in the following medium:

Sodium Citrate	1 mM
Potassium Phosphate, pH 7.9	5 mM
Ammonium Chloride	10 mM
Magnesium Sulfate	0.2 mM
Calcium Chloride	0.1 mM
Sodium Chloride	1 g/l
Yeast Extract	1 g/l
Tryptone	1 g/l
Glucose	2 g/l
Ferrous Sulfate	0.01 mM

(The pH was adjusted to 8.0 prior to autoclaving.)

A 10-liter fermentor was inoculated from a seed flask cultured overnight in the above medium at 70° C. A total of 600 ml from the seed flask was used to inoculate 10 liters of the same medium. The pH was controlled at 8.0 with ammonium hydroxide with the dissolved oxygen at 40%, with the temperature at 70° C., and with the stirring rate at 400 rpm. After growth of the cells, they were purified using the protocol (with slight modification) of Kaledin et al., supra, through the first five stages and using a different protocol for the sixth stage. All six steps were conducted at 4° C. The rate of fractionation on columns was 0.5 columns/hour and the volumes of radients during elution were 10 column volumes. An alternative and preferred purification protocol is provided in Example VI below.

Briefly, the above culture of the *T. aquaticus* cells was harvested by centrifugation after nine hours of cultivation, in late log phase, at a cell density of 1.4 g dry weight/1. Twenty grams of cells were resuspended in 80 ml of a buffer consisting of 50 mM Tris.HCl pH 7.5, 0.1 mM EDTA. Cells were lysed and the lysate was centrifuged for two hours at 35,000 rpm in a Beckman TI 45 rotor at 4° C. The supernatant was collected (fraction A) and the protein fraction precipitating between 45 and 75% saturation of ammonium sulfate was collected, dissolved in a buffer consisting of 0.2 M potassium phosphate buffer, pH 6.5, 10 mM 2-mercaptoethanol, and 5% glycerine, and finally dialyzed against the same buffer to yield fraction B.

Fraction B was applied to a 2.2×30-cm column of DEAE-cellulose, equilibrated with the above described buffer. The column was then washed with the same buffer and the fractions containing protein (determined by absorbance at 280 nm) were collected. The combined protein fraction was dialyzed against a second buffer, containing 0.01 M potassium phosphate buffer, pH 7.5, 10 mM 2-mercaptoethanol, and 5% glycerine, to yield fraction C.

Fraction C was applied to a 2.6×21-cm column of hydroxyapatite, equilibrated with a second buffer. The column was then washed and the enzyme was eluted with a linear gradient of 0.01–0.5 M potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 5% glycerine. Fractions containing DNA polymerase activity (90–180 mM potassium phosphate) were combined, concentrated four-fold using an Amicon stirred cell and YM10 membrane, and dialyzed against the second buffer to yield fraction D.

Fraction D was applied to a 1.6×28-cm column of DEAE-cellulose, equilibrated with the second buffer. The column was washed and the polymerase was eluted with a linear gradient of 0.01–0.5M potassium phosphate in the second buffer. The fractions were assayed for contaminating endonuclease(s) and exonuclease(s) by electrophoretically detecting the change in molecular weight of phage λ DNA or supercoiled plasmid DNA after incubation with an excess of DNA polymerase (for endonuclease) and after treatment with a restriction enzyme that cleaves the DNA into several fragments (for exonuclease). Only those DNA polymerase fractions (65–95 mM potassium phosphate) having minimal nuclease contamination were pooled. To the pool was added autoclaved gelatin in an amount of 250 μg/ml, and dialysis was conducted against the second buffer to yield Fraction E.

Fraction E was applied to a phosphocellulose column and eluted with a 100 ml gradient (0.01–0.4 M KCl gradient in 20 mM potassium phosphate buffer pH 7.5). The fractions were assayed for contaminating endo/exonuclease(s) as described above as well as for polymerase activity (by the method of Kaledin et al.) and then pooled. The pooled fractions were dialyzed against the second buffer, then concentrated by dialysis against 50% glycerine and the second buffer.

The molecular weight of the polymerase was determined by SDS PAGE. Marker proteins (Bio-Rad low molecular weight standards) were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Preliminary data suggest that the polymerase has a molecular weight of about 86,000–90,000 daltons, not 62,000–63,000 daltons reported in the literature (e.g., by Kaledin et al.).

The polymerase was incubated in 50 μl of a mixture containing either 25 mM Tris-HCl pH 6.4 on pH 8.0, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 nmoles each of dGTP, dATP, and TTP, and 0.5 μCi (^{3}H) dCTP, 8 μg "activated" calf thymus DNA, and 0.5–5 units of the polymerase. "Activated" DNA is a native preparation of DNA after partial hydrolysis with DNase I until 5% of the DNA was transferred to the acid-soluble fraction. The reaction was conducted at 70° C. for 30 minutes, and stopped by adding 50 μl of a saturated aqueous solution of sodium pyrophosphate containing 0.125 M EDTA-Na_2. Samples were processed and activity was determined as described by Kaledin et al., supra.

The results showed that at pH 6.4 the polymerase was more than one-half as active as at pH 8.0. In contrast, Kaledin et al. found that at pH about 7.0, the enzyme therein had 8% of the activity at pH 8.3. Therefore, the pH profile for the thermostable enzyme herein is broader than that for the Kaledin et al. enzyme.

Finally, when only one or more nucleotide triphosphates were eliminated from a DNA polymerase assay reaction mixture, very little, if any, activity was observed using the enzyme herein, and the activity was consistent with the expected value, and with an enzyme exhibiting high fidelity. In contrast, the activity observed using the Kaledin et al. (supra) enzyme is not consistent with the expected value, and suggests misincorporation of nucleotide triphosphate(s).

IV. Amplification Reaction

One microgram of the genomic DNA described above was diluted in an initial 100 μl aqueous reaction volume containing 25 mM Tris.HCl buffer (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 200 μg/ml gelatin, 1 μM of primer PC03, 1 μM of primer PC04, 1.5 mM dATP, 1.5 mM dCTP, 1.5 mM dGTP and 1.5 mM TTP. The sample was heated for 10 minutes at 98° C. to denature the genomic DNA, then cooled to room temperature. Four microliters of the polymerase from *Thermus aquaticus* was added to the reaction mixture and overlaid with a 100 μl mineral oil cap. The sample was then placed in the aluminum heating block of the liquid handling and heating instrument described in copending U.S. application Ser. No. 833,368 filed Feb. 25, 1986, the disclosure of which is incorporated herein by reference.

The DNA sample underwent 20 cycles of amplification in the machine, repeating the following program cycle:

(1) heating from 37° C. to 98° C. in heating block over a period of 2.5 minutes; and

(2) cooling from 98° C. to 37° C. over a period of three minutes to allow the primers and DNA to anneal.

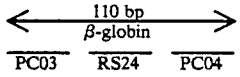
After the last cycle, the sample was incubated for an additional 10 minutes at 55° C. to complete the final extension reaction.

V. Synthesis and Phosphorylation of Oligodeoxyribonucleotide Probes

A labeled DNA probe, designated RS24, of the following sequence was prepared:

5'-*CCCACAGGGCAGTAACG-
GCAGACTTCTCCTCAGGAGTCAG-3'
(RS24)

where * indicates the label. This probe is 40 bases long, spans the fourth through seventeenth codons of the gene, and is complementary to the normal β-globin allele (β^A). The schematic diagram of primers and probes is given below:



This probe was synthesized according to the procedures described in Section I of Example 1. The probe was labeled by contacting 20 pmole thereof with 4 units of T4 polynucleotide kinase (New England Biolabs) and about 40 pmole γ-^{32}P-ATP (New England Nuclear, about 7000 Ci/mmole) in a 40 μl reaction volume containing 70 mM Tris buffer (pH 7.6), 10 mM $MgCl_2$, 1.5 mM sperine, and 10 mM dithiothreitol for 60 minutes at 37° C. The total volume was then adjusted to 100 μl with 25 mM EDTA and the probe purified according to the procedure of Maniatis et al., *Molecular Cloning* (1982), 466–467 over a 1 ml Bio Gel P-4 (BioRad) spin dialysis column equilibrated with Tris-EDTA (TE) buffer (10 mM Tris buffer, 0.1 mM EDTA, pH 8.0). TCA precipitation of the reaction product indicated that for RS24 the specific activity was 4.3 μCi/pmole and the final concentration was 0.118 pmole/μl.

VI. Dot Blot Hybridizations

Four microliters of the amplified sample from Section IV and 5.6 μl of appropriate dilutions of β-globin plasmid DNA calculated to represent amplification efficiencies of 70, 75, 80, 85, 90, 95 and 100% were diluted with 200 μl 0.4 N NaOH, 25 mM EDTA and spotted onto a Genatran 45 (Plasco) nylon filter by first wetting the filter with water, placing it in a Bio-Dot (Bio-Rad, Richmond, CA) apparatus for preparing dot blots which holds the filters in place, applying the samples, and rinsing each well with 0.1 ml of 20×SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA), as disclosed by Reed and Mann, *Nucleic Acids Research*, 13, 7202–7221 (1985). The filters were then removed, rinsed in 20×SSPE, and baked for 30 minutes at 80° C. in a vacuum oven.

After baking, each filter was then contacted with 16 ml of a hybridization solution consisting of 3×SSPE, 5×Denhardt's solution (1×=0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin,, 0.2 mM Tris, 0.2 mM EDTA, pH 8.0), 0.5% SDS and 30% formamide, and incubated for two hours at 42° C. Then 2 pmole of probe RS24 was added to the hybridization solution and the filter was incubated for two minutes at 42° C.

Finally, each hybridized filter was washed twice with 100 ml of 2×SSPE and 0.1% SDS for 10 minutes at room temperature. Then the filters were treated once with 100 ml of 2×SSPE, 0.1% SDS at 60° C. for 10 minutes.

Each filter was then autoradiographed, with the signal readily apparent after two hours.

VII. Discussion of Autoradiogram

The autoradiogram of the dot blots was analyzed after two hors and compared in intensity to standard serial dilution β-globin reconstructions prepared with HaeIII/MaeI-digested pBR:β^A, where β^A is the wild-type allele, as described in Saiki et al., *Science*, supra. Analysis of the reaction product indicated that the overall amplification efficiency was about 95%, corresponding to a 630,000-fold increase in the β-globin target sequence.

EXAMPLE II

I. Amplification Reaction

Two 1 μg samples of genomic DNA extracted from the Molt 4 cell line as described in Example I were each diluted in a 100 μl reaction volume containing 50 mM KCl, 25 mM Tris.HCl buffer pH 8.0, 10 mM $MgCl_2$, 1 μM of primer PC03, 1 μM of primer PC04, 200 μg/ml gelatin, 10% dimethylsulfoxide (by volume), and 1.5 mM each of dATP, dCTP, dGTP and TTP. After this mixture was heated for 10 minutes at 98° C. to denature the genomic DNA, the samples were cooled to room temperature and 4 μl of the polymerase from *Thermus aquaticus* described in Example I was added to each sample. The samples were overlaid with mineral oil to prevent condensation and evaporative loss. One of the samples was placed in the heating block of the machine described in Example I and subjected to 25 cycles of amplification, repeating the following program cycle:

(1) heating from 37° to 93° C. over a period of 2.5 minutes.,

(2) cooling from 93° C. to 37° C. over a period of three minutes to allow the primers and DNA to anneal; and

(3) maintaining at 37° C. for two minutes.

After the last cycle the sample was incubated for an additional 10 minutes at 60° C. to complete the final extension reaction.

The second sample was placed in the heat-conducting container of the machine, described in more detail in copending U.S. Ser. No. 899,061 filed Aug. 22, 1986. The heat-conducting container is attached to Paltier heat pumps which adjust the temperature upwards or downwards and a microprocessor controller to control automatically the amplification sequence, the temperature levels, the temperature ramping and the timing of the temperature.

The second sample was subjected to 25 cycles of amplification, repeating the following program cycle:

(1) heating from 37° to 95° C. over a period of three minutes;

(2) maintaining at 95° C. for 0.5 minutes to allow denaturation to occur;

(3) cooling from 95° to 37° C. over a period of one minute; and

(4) maintaining at 37° C. for one minute.

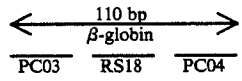
II. Analysis

Two tests were done for analysis, a dot blot and an agarose gel analysis.

For the dot blot analysis, a labeled DNA probe, designated RS18, of the following sequence was prepared.

5'-*CTCCTGAGGAGAAGTCTGC-3' (RS18)

where * indicates the label. This probe is 19 bases long, spans the fourth through seventeenth codons of the gene, and is complementary. to the normal β-globin allele (β^A). The schematic diagram of primers and probes is given below:



This probe was synthesized according to the procedures described in Section I of Example 1. The probe was labeled by contacting 10 pmole thereof with 4 units of T4 polynucleotide kinase (New England Biolabs) and about 40 pmole γ-^{32}P-ATP (New England Nuclear, about 7000 Ci/mmole) in a 40 μl reaction volume containing 70 mM Tris.HCl buffer (pH 7.6 , 10 mM $MgCl_2$, 1.5 mM spermine and 10 mM dithiothreitol for 60 minutes at 37° C. The total volume was then adjusted to 100 μl with 25 mM EDTA and purified according to the procedure of Maniatis et al., supra, p. 466–467 over a 1 ml Bio Gel P-4 (BioRad) spin dialysis column equilibrated with Tris-EDTA (TE) buffer (10 mM Tris.HCl buffer, 0.1 mM EDTA, pH 8.0). TCA precipitation of the reaction product indicated that for RS18 the specific activity was 4.6 μCi/pmole and the final concentration was 0.114 pmole/μl.

Five microliters of the amplified sample from Section I and of a sample amplified as described above except using the Klenow fragment of *E. coli* DNA Polymerase I instead of the thermostable enzyme were diluted with 195 μl 0.4 N NaOH, 25 mM EDTA and spotted onto two replicate Genatran 45 (Plascol nylon filters by first wetting the filters with water, placing them in a Bio-Dot (Bio-Rad, Richmond, CA) apparatus for preparing dot blots which holds the filters in place, applying the samples, and rinsing each well with 0.4 ml of 20×SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA), as disclosed by Reed and Mann, supra. The filters were then removed, rinsed in 20×SSPE, and baked for 30 minutes at 80° C. in a vacuum oven.

After baking, each filter was then contacted with 6 ml of a hybridization solution consisting of 5×SSPE, 5'Denhardt's solution (1×=0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 0.2 mM Tris, 0.2 mM EDTA, pH 8.0) and 0.5% SDS, and incubated for 60 minutes at 55° C. Then 5 ul of probe RS18 was added to the hybridization solution and the filter was incubated for 60 minutes at 55° C.

Finally, each hybridized filter was washed twice with 100 ml of 2×SSPE and 0.1% SDS for 10 minutes at room temperature. Then the filters were treated twice more with 100 ml of 5×SSPE, 0.1% SDS at 60° C. for 1) one minute and 2) three minutes, respectively.

Each filter was then autoradiographed, with the signal readily apparent after 90 minutes.

In the agarose gel analysis, 5 μl each amplification reaction was loaded onto 4% NuSieve/0.5% agarose gel in 1×TBE buffer (0.089 M Tris, 0.089 M boric acid, and 2 mM EDTA) and electrophoresed for 60 minutes at 100V. After staining with ethidium bromide, DNA was visualized by UV fluorescence.

The results show that the machines used in Example I and this example were equally effective in amplifying the DNA, showing discrete high-intensity 110-base pair bands of similar intensity, corresponding to the desired sequence, as well as a few other discrete bands of much lower intensity. In contrast, the amplification method as described in Example I of now abandoned U.S. application Ser. No. 839,331 filed Mar. 13, 1986, supra, which involves reagent transfer after each cycle using the Klenow fragment of *E. coli* Polymerase I, gave a DNA smear resulting from the non-specific amplification of many unrelated DNA sequences.

It is expected that similar improvements in amplification and detection would be achieved in evaluating HLA-DQ, DR and DP regions.

If in the above experiments the amplification reaction buffer contains 2 mM $MgCl_2$ instead of 10 mM $MgCl_2$ and 150–200 μM of each nucleotide rather than 1.5 mM of each, and if the lower temperature of 37° C. is raised to 45°–58° C. during amplification, better specificity and efficiency of amplification occurs. Also, DMSO was found not necessary or preferred for amplification.

EXAMPLE III

Amplification and Cloning

For amplification of a 119-base pair fragment on the human β-globin gene, a total of 1 microgram each of human genomic DNA isolated from the Molt 4 cell line or from the GM2064 cell line (representing a homozygous deletion of the β- and δ-hemoglobin region and available from the Human Genetic Mutant Cell Depository, Camden, NJ) as described above was amplified in a 100 μl reaction volume containing 50 mM KCl, 25 mM Tris.HCl pH 8, 10 mM $MgCl_2$, 200 μg/ml gelatin, 5 mM 2-mercaptoethanol, 1.5 mM each of dATP, dCTP, TTP, and dGTP, and 1 μM of each of the following primers:

5'-CTTCTGcagCAACTGTGTTCACTAGC-3' (GH18)

5'-CACaAgCTTCATCCACGTTCACC-3' (GH19)

where lower case letters denote mismatches from wild-type sequence to create restriction enzyme sites. GH18 is a 26-base oligonucleotide complementary to the negative strand and contains an internal PstI site. GH19 is a 29-base oligonucleotide complementary to the plus strand and contains an internal HindIII recognition sequence. These primers were selected by first screening the regions of the gene for homology to the PstI and HindIII restriction sites. The primers were then prepared as described in Example I.

The above reaction mixtures were heated for 10 minutes at 95° C. and then cooled to room temperature. A total of 4 μl of the polymerase described in Example I was added to each reaction mixture, and then each mixture was overlayed with mineral oil. The reaction mixtures were subjected to 30 cycles of amplification with the following program:

2.5 min. ramp, 37° to 98° C.
3 min. ramp, 98° to 37° C.
2 min. soak, 37° C.

After the last cycle, the reaction mixtures were incubated for 20 minutes at 65° C. to complete the final extension. The mineral oil was extracted with chloroform and the mixtures were stored at −20° C.

A total of 10 μl of the amplified product was digested with 0.5 μl M13mp10 cloning vector, which is publicly available from Boehringer-Mannheim, in a 50 μl volume containing 50 mM NaCl, 10 mM Tris.HCl, pH 7.8, 10 mM $MgCl_2$, 20 units PstI and 26 units HindIII for 90 minutes at 37° C. The reaction was stopped by freezing at −20° C. The volume was adjusted to 110 μl with TE buffer and loaded (100 μl) onto a 1 ml BioGel P-4 spin dialysis column. One 0.1 ml fraction was collected and ethanol precipitated.

(At this point it was discovered that there was β-globin amplification product in the GM2064 sample. Subsequent experiments traced the source of contamination to the primers, either GH18 or GH19. Because no other source of primers was available, the experiment was continued with the understanding that some cloned sequences would be derived from the contaminating DNA in the primers.)

The ethano pellet was resuspended in 15 μl water, then adjusted to 20 μl volume containing 50 mM Tris.HCl, pH 7.8, 10 mM $MgCl_2$, 0.5 mM ATP, 10 mM dithiothreitol, and 400 units ligase. This mixture was incubated for three hours at 16° C.

Ten microliters of ligation reaction mixture containing molt 4 DNA was transformed into *E. coli* strain JM103 competent cells, which are publicly available from BRL in Bethesda, MD. The procedure followed for preparing the transformed strain is described in Messing, J. (1981) *Third Cleveland Symposium on Macromolecules:Recombinant DNA,* ed. A. Walton, Elsevier, Amsterdam, 143–163. A total of 651 colorless plaques (and 0 blue plaques) were obtained. Of these, 119 had a (+)strand insert (18%) and 19 had a (−)-strand insert (3%). This is an increase of almost 20-fold over the percentage of β-globin positive plaques among the primer-positive plaques from the amplification technique using klenow fragment of *E. coli* Polymerase I, where the reaction proceeded for two minutes at 25° C., after which the steps of heating to 100° C. for two minutes, cooling, adding klenow fragment, and reacting were repeated nine times. These results confirm the improved specificity of the amplification reaction employing the thermostable enzyme herein.

In a later cloning experiment with GM2064 and the contaminated primers, 43 out of 510 colorless plaques (8%) had the (+)- strand insert. This suggests that approximately one-half of the 119 clones from Molt 4 contain the contaminant sequence.

Ten of the (+)- strand clones from Molt 4 were sequenced. Five were normal wild-type sequence and five had a single C to T mutation in the third position of the second codon of the gene (CAC to CAT). Four of the contaminant clones from GM2064 were sequenced and all four were normal.

Restriction site-modified primers may also be used to amplify and clone and partially sequence the human N-ras oncogene and to clone base pair segments of the HLA DQ-α, DQ-β and DR-β genes using the above technique.

Again, if the concentrations of $MgCl_2$ and nucleotides are reduced to 2 mM and 150–200 μM, respectively, and the minimum cycling temperature is increased from 37° C. to 45°–58° C., the specificity and efficiency of the amplification reaction can be increased.

EXAMPLE IV

Gene Retrieval

A. Identification of a DNA Sequence Probe for the Taq Polymerase Gene.

A specific DNA sequence probe for the Taq pol gene was obtained following immunological screening of a λgt11 expression library. *T. aquaticus* DNA was digested to completion with AluI, ligated with EcoRI 12-mer linkers (CCGGAATTCCGG, New England Biolabs), digested with EcoRI and ligated with dephosphorylated, EcoRI-digested λgt11 DNA (Promega Biotech). The ligated DNA was packaged (Gigapack Plus, Strategene) and transfected into *E. coli* K-12 strain Y1090 (provided by R. Young).

The initial library of 2×10^5 plaques was screened (Young, R. A., and R. W. Davis (1983) Science, 222:778–782) with a 1:2000 dilution of a rabbit polyclonal antiserum raised to purified Taq polymerase (see Examples I and VI). Candidate plaques were replated at limiting dilution and rescreened until homogeneous (~3 cycles). Phage were purified from candidate plaques which failed to react with preimmune serum and reacted with immune serum.

Candidate phge were used to lysogenize *E. coli* K-12 strain Y1089 (R. Young). Lysogens were screened for the production of an IPTG inducible fusion protein (larger than β-galactosidase) which reacted with the Taq polymerase antiserum. Solid phase, size-fractionated fusion proteins were used to affinity purify epitope-specific antibodies from the total polyclonal antiserum (Goldstein, L. S. B., et al. (1986) J. Cell Biol. 102:2076–2087).

The "fished", epitope-selected antibodies were used, in turn, in a Western analysis to identify which λgt11 phage candidates encoded DNA sequences uniquely specific to Taq polymerase. One λgt11 phage candidate, designated λgt:1, specifically selected antibodies from the total rabbit polyclonal Taq polymerase antiserum which uniquely reacted with both purified Taq polymerase and crude extract fractions containing Taq polymerase. This phage, λgt:1, was used for further study.

The ~115 bp EcoRI-adapted AluI fragment of *Thermus aquaticus* DNA was labeled (Maniatis et al., supra) to generate a Taq polymerase-specific probe. The probe was used in Southern analyses and to screen a *T. aquaticus* DNA random genomic library.

B. Construction and Screening of a Thermus Aquaticus Random Genomic Library.

Lambda phage Charon 35 (Wilhelmine, A. M. et al., supra) was annealed and ligated via its cohesive ends, digested to completion with BamHI, and the annealed arms were purified from the "stuffer" fragments by potassium acetate density gradient ultracentrifugation (Maniatis, et al., supra). *T. aquaticus* DNA was partially digested with Sau3A and the 15–20 kb size fraction purified by sucrose density gradient ultracentrifugation. The random genomic library was - constructed by ligating the target and vector DNA fragments at a 1:1 molar ratio. The DNA was packaged and transfected into *E. coli* K-12 strains LE392 or K802. A library of >20,000 initial phage containing >99% recombinants was amplified on *E. coli* K-12 strain LE392.

The CH35 Taq genomic phage library was screened (Maniatis et al., supra) with the radiolabeled EcoRI insert of λgt11:1. Specifically hybridizing candidate phage plaques were purified and further analyzed. One phage, designated Ch35::4–2, released ≧four *T. aquaticus* DNA fragments upon digestion with HindIII (~8.0, 4.5, 0.8, 0.58 kb)

The four HindIII *T. aquaticus* DNA fragments were ligated with HindIII digested plasmid BSM13+ (3.2 kb, Vector Cloning Systems, San Diego) and individually cloned following transformation of *E. coli* K-12 strain DG98.

The ~8.0 kb HindIII DNA fragment from CH35::4-2 was isolated in plasmid pFC82 (11.2 kb), while the 4.5 kb HindIII DNA fragment from CH35::4-2 was isolated in plasmid pFC83 (7.7 kb).

E. coli strain DG98 harboring pFC82 was shown to contain a thermostable, high temperature DNA poly-

merase activity (Table 1). In addition, these cells synthesize a new ~60 kd molecular weight polypeptide which is immunologically related to Taq DNA polymerase.

The Taq polymerase coding region of the 8.0 kb HindIII DNA fragment was further localized to the lac-promoter proximal 2.8 kb HindIII to Asp718 portion of the 8.0 kb HindIII fragment. This region was subcloned to yield plasmid pFC85 (6.0 kb). Upon induction with IPTG, *E. coli* DG98 cells harboring plasmid pFC85 synthesize up to 100-fold more thermostable, Taq polymerase-related activity (Table 1) than the original parent clone (pFC82/DG98). While cells harboring pFC85 synthesize a significant amount of a thermostable DNA polymerase activity, only a portion of the Taq pol DNA sequence is translated, resulting in the accumulation of a ~60 kd Taq polymerase-related polypeptide.

TABLE 1

Expression of a Thermostable DNA Polymerase Activity in *E. coli*#

Sample	Units*/ml IPTG	Units*/ml +IPTG
BSM13/DG98-	—	0.02
pFC82/DG98	2.2	2.7
pFC85/DG98	11.9	643.8

#Cells were grown to late log phase (+/−IPTG, 10 mM), harvested, sonicated, heated at 75° C. for 20 minutes, centrifuged and the clarified supernatant assayed at 70° C. for DNA polymerase activity.
*1 unit = 1 nM dCTP incorporated in 30 minutes.

EXAMPLE V

Expression of Taq Polymerase

The thermostable gene of the present invention can be expressed in any of a variety of bacterial expression vectors including DG141 (ATCC 39588) and $_pP_LN_{RBS}$ATG, a vector disclosed in commonly owned, U.S. Pat. No. 4,711,845, filed Dec. 24, 1984 (Gelfand et al.), the disclosure of which is incorporated herein by referenee. Both of these host vectors are $_p$BR322 derivatives that have either a sequence containing a tryptophan promoter-operator and ribosome binding site with an operably linked ATG start codon (DG141) or a sequence containing the lambda P_L promoter and gene N ribosome binding site operably linked to an ATG start codon ($_pP_LN_{RBS}$ATG). Either one of these host vectors may be restricted with SacI, and blunt ended with Klenow or Sl nuclease to construct a convenient restriction site for subsequent insertion of the Taq polymerase gene.

The full-length Taq polymerase gene was constructed from the DNA insert fragments subcloned into plasmids pFC83 and pFC85 as follows. Vector BSM13+ (commercially available from Vector Cloning Systems, San Diego, CA) was digested at the unique HindIII site, repaired with Klenow and dNTPs, and ligated with T4 DNA ligase to a BglII octanucleotide linker, 5'-CAGATCTG-3' (New England Biolabs), and transformed into *E. coli* strain DG98. Plasmids were isolated from Amp^R lacZα^+ transformants. One of the clones was digested with BglII and Asp718 restriction enzymes, and the large vector fragment purified by gel electrophoresis.

Next, plasmid pFC83 was digested with BglII and HindIII and the ~750 base pair fragment was isolated. Plasmid pFC85 was digested with HindIII and Asp718 and the ~2.8 kb fragment isolated and joined in a three-piece ligation to the ~750 base pair BglII-HindIII fragment from pFC83 and the Bg1II-Asp718 vector fragment of BSM13+. This ligation mixture was used to transform E. coli strain DG98 (ATCC 39,768 deposited July 13, 1984) from which Amp^R colonies were selected and an ~6.75 kilobase plasmid (pLSGI) was isolated. Isopropyl-β-D-thiogalactoside (IPTG)-induced DG98 cells harboring pLSGI synthesized Taq DNA polymerase indistinguishable in size from the native enzyme isolated from *T. aquaticus*. Plasmid pLSG1 can then be used to generate a single strand DNA template according to the procedure recommended by Vector Cloning Systems.

Oligonucleotide-directed mutagenesis (see Zoller and Smith, *Nuc. Acids Res.* (1982) 10:6487–6500) can then be used to introduce an SphI restriction site as part of the ATG start codon (upstream of the internal HindIII site in the coding sequence of the Taq polymerase gene). Similarly, a BglII site can be introduced after the carboxyl-terminus of the gene (~0.7 kb upstream from the Asp718 site) to facilitate subcloning of the Taq polymerase gene into an expression vector. After the site-directed mutagenesis is performed, the gene can be isolated from the BSM13+ vector on an ~3.2 kb SphI-BstEII restriction fragment, treated with Klenow fragment and all four dNTPs, and inserted with T4 DNA ligase (blunt-end conditions) into either one of the aforementioned expression vectors, which have been digested with SacI, repaired with Klenow and dNTPs, and treated with calf intestine phosphatase to generate dephosphorylated blunt ends. This ligation mixture is used to transform *E. coli* DG116 and the resulting transformants are screened for production of Taq polymerase. Expression of the enzyme can be confirmed by Western immunoblot analysis and activity analysis.

A greater proportion of the Taq polymerase gene contained within the ~2.8 kb HindIII-Asp718 fragment of plasmid pFC85 can be expressed using, for example, plasmid $_pP_LN_{RBS}$ATG, by operably linking the amino-terminal HindIII restriction site encoding the Taq pol gene to an ATG initiation codon. The product of this fusion upon expression will yield an ~66,000–68,000 dalton truncated polymerase.

This specific construction can be made by digesting plasmid pFC85 with HindIII and treating with Klenow fragment in the presence of dATP, dGTP and dCTP. The resulting fragment is treated further with Sl nuclease to remove any single-stranded extensions, and the resulting DNA digested with Asp718 and treated with Klenow fragment in the presence of all four dNTPs. The recovered fragment can be ligated

using T4 DNA ligase to dephosphorylated plasmid $_pP_LN_{RBS}$ATG, which had been digested with SacI and treated with Klenow fragment in the presence of dGTP to construct an ATG blunt end. This ligation mixture can then be used to transformed *E. coli* DGI16 and the transformants screened for production of Taq polymerase. Again, expression can be confirmed by Western immunoblot analysis and activity analysis.

EXAMPLE VI

Purification

The thermostable polymerase may be purified directly from a culture of *Thermus aquaticus* following the example disclosed below or, alternatively, from a bacterial culture containing the recombinantly pro-

duced enzyme with only minor modifications necessary in the preparation of the crude extract.

After harvesting by centrifugation, 60 grams of cells were resuspended in 75 ml of a buffer consisting of 50 mM Tris-Cl pH 8, 1 mm EDTA. Cells were lysed in a French Press at 14,000–16,000 PSI after which 4 volumes (300 ml) of additional Tris-EDTA were added. Buffer A (β-mercaptoethanol to 5 mM and NP-40 and Tween 20 to 0.5% (v/v) each) was added and the solution was sonicated thoroughly while cooling. The resultant homogeneous suspension was diluted further with Buffer A such that the final volume was 7.5–8 times the starting cell weight; this was designated Fraction I.

The polymerase activity in Fraction I and subsequent fractions was determined in a 50 μl mixture containing 0.025 M TAPS-HCl pH 9.4 (20° C.) 0.002 M $MgcL_2$, 0.05 M KCl, 1 mM 2-mercaptoethanol, 0.2 mM each dGTP, dATP, TTP, 0.1 mM dCTP [α-^{32}P, 0.05 Ci/mM], 12.5 μg "activated" salmon sperm DNA and 0.01–0.2 units of the polymerase (diluted in 10 mM Tris-HCl, pH 8, 50 mM KCl, 1 mg/ml autoclaved gelatin, 0.5% NP-40, 0.5% Tween 20, and 1 mM 2-mercaptoethanol). One unit corresponds to 10 nM product in 30 minutes. "Activated" DNA is a native preparation of DNA after partial hydrolysis with DNase I until 5% of the DNA was transferred to the acid-soluble fraction. The reaction was conducted at 74° C. for 10 minutes and then 40 μl was transferred to 1.0 ml of 50 μg/ml carrier DNA in 2 mM EDTA at 0° C. An equal volume (1.0 ml) of 20% TCA, 2% sodium pyrophosphate was added. After 15–20 minutes at 0° C. the samples were filtered through Whatman GF/C discs and extensively washed with cold 5% TCA-1% pyrophosphate, followed by cold 95% ethanol, dried and counted.

Fraction I was centrifuged for two hours at 35,000 rpm in a Beckman TI 45 rotor at 2° C. and the collected supernatant was designated Fraction II.

The Taq polymerase activity was precipitated with Polymin P (BRL, Gaithersburg, MD) (10%, w/v, adjusted to pH 7.5 and autoclaved) after the minimum amount of Popymin P necessary to precipitate 90–95% of the activity was determined, which amount was generally found to be between 0.25% and 0.3% final volume.

An appropriate level of Polymin P was added slowly to Fraction II while stirring for 15 minutes at 0° C. This solution was centrifuged at 13,000 rpm for 20 minutes in a Beckman JA 14 rotor at 2° C. The supernatant was assayed for activity and the pellet was resuspended in 1/5 volume of 0.5X Buffer A (diluted 1:2 with H_2O). This suspension was recentrifuged and the pellet resuspended in ¼ volume of Buffer A containing 0.4 M KCl. This suspension was homogenized thoroughly and left overnight at 4° C. The homogenate was centrifuged as above and the collected supernatant designated Fraction III.

The protein fraction was collected by "precipitation" at 75% saturation of ammonium sulfate, centrifuged (at 27,000 rpm, SW27 rotor, 30 minutes) and the floating pellicle was resuspended in 50 mM Tris-Cl pH 8, 1 mM EDTA. These steps were repeated and the protein suspension was dialyzed extensively with P-cell buffer (20 mM KPO_4 pH 7.5, 0.5 mM EDTA, 5 mM β-mercaptoethanol, 5% (w/v) glycerol, 0.5% (v/v) NP-40 and Tween 20) containing 80 mM KCl.

The dialysate was transferred to a centrifuge bottle to which was added any recovered protein from sacks rinsed with the P-cell buffer containing 80 mM KCl. Centrifugation was performed at 20,000×g and the time was reduced to 15 minutes. The supernatant was saved and any pellet remaining was washed, extracted with P-cell buffer and 80 mM KCl, and recentrifuged. The supernatants were then combined to form Fraction IV.

Fraction IV was applied to a 2.2×22-cm column of phosphocellulose, equilibrated with the P-cell buffer containing 80 mM KCl. The column was washed (2.5–3 column volumes) with the same buffer and the protein eluted using a linear gradient of 80 to 400 mM KCl in P-cell buffer. Fractions containing DNA polymerase activity (~0.18–0.20 M KCl) were pooled and concentrated 3–4 fold on an Amicon stirred cell and YM30 membrane. The cell was rinsed with the P-cell buffer without KCl and added to the fraction concentrate (0.15 M KCl adjusted final volume) to form Fraction V.

Fraction V was applied to a 5 ml Heparin Sepharose CL-6B column (Pharmacia) equilibrated with P-cell buffer and 0.15 M KCl. The column was washed with 0.15 M KCl buffer (3–4 column volumes) and the protein eluted with a linear gradient from 0.15 to 0.65 M KCl in P-cell buffer. A 1:10 dilution into diluent without gelatin was made for SDS-PAGE analysis and a subsequent 1:20 dilution into diluent with 1 mg/ml gelatin was made for use in enzyme assays. The activity fractions (eluting at ~0.3 M KCl) were assayed on supercoiled DNA template for specific and non-specific endonucleases/topoisomerase by electrophoretically detecting the change in molecular weight of supercoiled plasmid DNA after incubation with an excess of DNA polymerase. Exonuclease contamination was detected following incubation with small linear DNA fragments. In peak fractions, an 88 kd protein was found to be the major band. The major pool, designated Fraction VI, had the highest polymerase activity with minimal detectable endonuclease activity when this pool was assayed for 30 minutes at 55° C. with ~3–5 polymerase units/600 ng DNA.

Fraction VI was dialyzed against 10 mM KPO_4 pH 7.5, 5 mM β-mercaptoethanol, 5% glycerol, 0.2% NP-40, and 0.2% Tween 20 (HA buffer). The dialyzed sample was applied to a 3 ml column of hydroxyapatite and the enzyme eluted with a linear gradient of 10 to 250 mM KPO_4 pH 7.5, HA buffer. DNA polymerase activity began to elute at 75 mM KPO_4 with the peak at 100 mM KPO_4. Active peak fractions were assayed at 1:100–1:300 dilution. As in the prior chromatography step, a 1:10 dilution in diluent was prepared without gelatin for SDS-PAGE analysis. Fractions with no significant endonuclease or double-strand exonuclease when assayed at 55° C. with 5 polymerase units were pooled and designated Fraction VII.

Fraction VII was dialyzed against a solution of 25 mM sodium acetate pH 5.2, 5% glycerol, 5 mM β-mercaptoethanol, 0.1 mM EDTA, 0.1% NP-40, and 0.1% Tween 20, adjusted to pH 5 at room temperature. The dialyzed sample was applied to a 2 ml DEAE-Tris-Acryl-M (LKB) column pre-equilibrated and subsequently washed with the same buffer. The fraction containing polymerase activity that did not adhere to the column was pooled and adjusted to 50 mM NaCl in the same buffer to yield Fraction VIII.

Fraction VIII was applied to a 2 ml CM-Tris-Acryl M (LKB) column equilibrated with the same buffer (25 mM sodium acetate, 50 mM NaCl, 5% glycerol, 0.1 mM EDTA, 0.1% NP-40, and 0.1% Tween 20). The column was washed with 4–5 column volumes of the same

buffer an the enzyme eluted with a linear gradient from 50 to 400 mM NaCl in sodium acetate buffer. The polymerase activity peak eluted ~0.15–0.20 M NaCl. The polymerase activity was assayed at 1:300 to 1:500 dilution with the first dilution 1:10 into diluent without gelatin for the SDS-PAGE analysis. An assay across the activity peak on supercoiled DNA templates for specific and non-specific endonuclease/topoisomerase using DNA polymerase assay salts (25 mM TAPS-HCl pH 9.4, 2.0 mM $MgCl_2$ and 50 mM KCl) at 74° C. was performed, as well as assays for nucleases on M13 as DNA and pBR322 fragments. Active fractions with no detectable nuclease(s) were pooled and run on a silver stained SDSPAGE mini gel. The results show a single ~88 kd band with a specific activity of ~250,000 units/mg.

This specific activity is more than an order of magnitude higher than that claimed for the previously isolated Taq polymerase and is at least an order of magnitude higher than that for *E. coli* polymerase I.

EXAMPLE VII

The Taq polymerase purified as described above in Example VI was found to be free of any contaminating Taq endonuclease and exonuclease activities. In addition, the Taq polymerase is preferably stored in storage buffer containing from about 0.1 to about 0.5% volume/volume of each non-ionic polymeric detergent employed. More preferably the storage buffer consists of 50% (v/v) glycerol, 100 mM KCl, 20 mM Tris-Cl pH 8.0, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 0.5% v/v NP-40, 0.5% v/v Tween 20, and 200 μg/ml gelatin, and is preferably stored at −20° C.

The stored Taq polymerase was diluted in a buffer consisting of 25 mM Tris Cl pH 8.0, 20 mM KCl, 1 mM β-mercaptoethanol, 0.5% NP-40, 0.5% Tween-20, and 500 μg/ml gelatin. A reaction buffer was then prepared containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM each dNTP, 1 μM each of the primers that define a 500 base pair target sequence on a control template from bacteriophage λ, and 2.0–2.5 units Taq polymerase/assay in a final volume of 100 μl. Template was added to the reaction buffer, the sample placed in a 0.5 ml polypropylene tube, and the sample topped with 100 μl of heavy white mineral oil to prevent evaporation.

At least a 10^5-fold amplification was achieved when the following conditions were employed, using 1 ng of control template (bacteriophage λ DNA) where the target sequence represented approximately 1% of the starting mass of DNA.

First the template mixture was denatured for one minute, 30 seconds at 94° C. by placing the tube in a heat bath. Then the tube was placed in a heat bath at 37° C. for two minutes. Then the tube was placed in a heat bath at 72° C. for three minutes, and then in the heat bath at 94° C. for one minute. This cycle was repeated for a total of 25 cycles. At the end of the 25th cycle, the heat denaturation step at 94° C. was omitted and replaced by extending the 72° C. incubation step by an additional three minutes. Following termination of the assay, the samples were allowed to cool to room temperature and analyzed as described in previous examples.

The template may be optimally amplified with a different concentration of dNTPs and a different amount of Taq polymerase. Also, the size of the target sequence in the DNA sample will directly impact the minimum time required for proper extension (72° C. incubation step). An optimization of the temperature cycling profile should be performed for each individual template to be amplified, to obtain maximum efficiency.

EXAMPLE VIII

Taq polymerase purified as described above in Example I was formulated for storage as described in the previous example, but without the non-ionic polymeric detergents. When assayed for activity as described in that example, the enzyme storage mixture was found to be inactive. When the NP-40 and Tween 20 were added to the storage buffer, the full enzyme activity was restored, indicating that the presence of the non-ionic detergents is necessary to the stability of the enzyme formulation.

EXAMPLE IX

Several 1 μg samples of human genomic DNA were subjected to 20–35 cycles of amplification as described in Example V, with equivalent units of either Klenow fragment or Taq polymerase, and analyzed by agarose gel electrophoresis and Southern blot. The primers used in these reactions, PC03 and PC04, direct the synthesis of a 110-bp segment of the human beta-globin gene. The Klenow polymerase amplifications exhibited the smear of DNA typically observed with this enzyme, the apparent cause of which is the non-specific annealing and extension of primers to unrelated genomic sequences under what were essentially non-stringent hybridization conditions (1×Klenow salts at 37° C.). Nevertheless, by. Southern blot a specific 110-bp beta-globin target fragment was detected in all lanes. A substantially different electrophoretic pattern was seen in the amplifications done with Taq polymerase where the single major band is the 110-bp target sequence. This remarkable specificity was undoubtedly due to the temperature at which the primers were extended.

Although, like Klenow fragment amplifications, the annealing step was performed at 37° C., the temperature of Taq-catalyzed reactions had to be raised to about 70° C. before the enzyme exhibited significant activity. During this transition from 37° to 70° C., poorly matched primer-template hybrids (which formed at 37° C.) disassociated so that by the time the reaction reached an enzyme-activating temperature, only highly complementary substrate was available for extension. This specificity also results in a greater yield of target sequence than similar amplifications done with Klenow fragment because the non-specific extension products effectively compete for the polymerase, thereby reducing the amount of 110-mer that can be made by the Klenow fragment.

EXAMPLE X

Amplification was carried out of a sample containing 1 μg Molt 4 DNA, 50 mM KCl, 10 mM Tris pH 8.3, 10 mM $MgCl_2$, 0.01% gelatin, 1 μM of each of the following primers (to amplify a 150 bp region):

5'-CATGCCTCTTTGCACCATTC-3'(RS79) and

5'-TGGTAGCTGGATTGTAGCTG-3'(RS80)

1.5 mM of each dNTP, and 5.0 units of Taq polymerase per 100 μl reaction volume. Three additional samples were prepared containing 2.5, 1.3, or 0.6 units of Taq

polymerase. The amplification was carried out in the temperature cycling machine described above using the following cycle, for 30 cycles:

from 70° to 98° C. for 1 minute
hold at 98° C. for 1 minute
from 98° C. to 35°, 45° or 55° C. for 1 minute
hold at 35°, 45° or 55° C. for 1 minute from 35°, 45° or 55° C. to 70° C. for 1 minute hold at 70° C. for 30 seconds

At 35° C. annealing temperature, the 2.5 units/100 μl Taq enzyme dilution gave the best-signal-to noise ratio by agarose gel electrophoresis over all other Taq polymerase concentrations. At 45° C., the 5 units/100 μl Taq enzyme gave the best signal-to-noise ratio over the other concentrations. At 55° C., the 5 units/100 μl Taq enzyme gave the best signal-to-noise ratio over the other concentrations and over the 45° C. annealing and improved yield. The Taq polymerase has more specificity and better yield at 55° C.

In a separate experiment the Molt 4 DNA was 10-fold serially diluted into the cell line GM2064 DNA, containing no β- or δ-globin sequences, available from the Human Genetic Mutant Cell Depository, Camden, New Jersey, at various concentrations representing varying copies per cell, and amplification was carried out on these samples as described in this example at annealing temperatures of 35° C. and 55° C. At 35° C., the best that can be seen by agarose gel electrophoresis is 1 copy in 50 cells. At 55° C., the best that can be seen is 1/5,000 cells (a 100-fold improvement over the lower temperature), illustrating the importance of increased annealing temperature for Taq polymerase specificity under these conditions.

In a third experiment, DNA from a cell line 368H containing HIV-positive DNA, available from B. Poiesz, State University of New York, Syracuse, NY, was similarly diluted into the DNA from the SCI cell line (deposited with ATCC on Mar. 19, 1985; an EBV-transformed β cell line homozygous for the sickle cell allele and lacking any HIV sequences at various concentrations representing varying copies per cell, and amplification was carried out as described in this Example at annealing temperatures of 35° C. and 55° C., using the primers SK38 and SK39, which amplify a 115 bp region of the HIV sequence:

5'-ATAATCCACCTATCCCAGTAG-GAGAAAT-3'(SK38) and

5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3'(SK39)

The results by agarose gel electrophoresis showed that only the undiluted 368H sample could be detected with the annealing temperature at 35° C., whereas at least a 10^{-2} dilution can be detected with the annealing temperature at 55° C., giving a 100-fold improvement in detection.

The following bacteriophage and bacterial strains were deposited with the Cetus Master Culture Collection, 1400 Fifty-Third Street, Emeryville, Calif., USA (CMCC) and with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md., USA (ATCC). These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from the date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between applicants and ATCC that assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Deposit Designation	CMCC No.	ATCC No.	Deposit
CH35:Taq #4–2	3125	ATCC 40336	5/28/87
E. coli DG98/ pFC83	3128	ATCC 67422	5/28/87
E. coli DG98/ pFC85	3127	ATCC 67421	5/28/87

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the cell lines deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any cell lines that are functionally equivalent are within the scope of this invention. The deposit of materials therein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor are the deposits to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

What is claimed is:

1. Purified thermostable Thermus aquaticus DNA polymerase that migrates on a denaturing polyacrylamide gel faster than phosphorylase B and more slowly than does bovine serum albumin and has an estimated molecular weight of 86,000–90,000 daltons when compared with a phosphorylase B standard assigned a molecular weight of 92,500 daltons

2. The polymerase of claim 1 that is isolated from *Thermus acquaticus.*

3. The polymerase of claim 1 that is isolated from a recombinant organism transformed with a vector that codes for the expression of *Thermis aquaticus* DNA polymerase.

* * * * *

US004889818C1

(12) EX PARTE REEXAMINATION CERTIFICATE (5797th)

United States Patent

Gelfand et al.

(10) Number: US 4,889,818 C1

(45) Certificate Issued: Jul. 3, 2007

(54) **PURIFIED THERMOSTABLE ENZYME**

(75) Inventors: **David H. Gelfand**, Oakland, CA (US); **Susanne Stoffel**, El Cerrito, CA (US); **Frances C. Lawyer**, Oakland, CA (US); **Randall K. Saiki**, Richmond, CA (US)

(73) Assignee: **Roche Molecular Systems, Inc.**, Branchburg, NJ (US)

Reexamination Request:
No. 90/006,184, Jan. 10, 2002
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Appl. No.: **07/063,509**
Filed: **Jun. 17, 1987**

Related U.S. Application Data

(63) Continuation-in-part of application No. 06/899,241, filed on Aug. 22, 1986, now abandoned.

(51) **Int. Cl.**
C12N 9/12 (2006.01)

(52) **U.S. Cl.** **435/194**; 536/23.2

(58) **Field of Classification Search** 435/194, 435/252.33; 536/23.2
See application file for complete search history.

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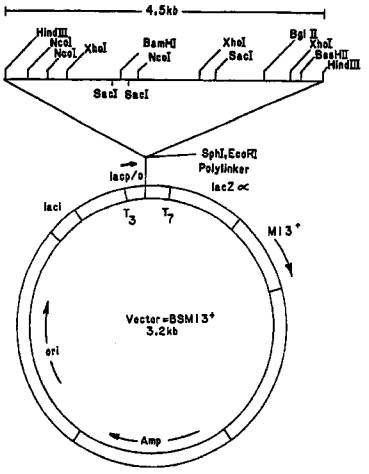
(Continued)

Primary Examiner—Nashaat T. Nashed

(57) **ABSTRACT**

A purified thermostable enzyme is obtained that has unique characteristics. Preferably the enzyme is isolated from the *Thermus aquaticus* species and has a molecular weight of about 86,000-90,000 daltons. The thermostable enzyme may be native or recombinant and may be used in a temperature-cycling chain reaction wherein at least one nucleic acid sequence is amplified in quantity from an existing sequence with the aid of selected primers and nucleotide triphosphates. The enzyme is preferably stored in a buffer of non-ionic detergents that lends stability to the enzyme.



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EX PARTE REEXAMINATION CERTIFICATE ISSUED UNDER 35 U.S.C. 307

THE PATENT IS HEREBY AMENDED AS INDICATED BELOW.

Matter enclosed in heavy brackets [ ] appeared in the patent, but has been deleted and is no longer a part of the patent; matter printed in italics indicates additions made to the patent.

AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

Claims **1**–**3** are cancelled.

New claim **4** is added and determined to be patentable.

4. The polymerase of claim 3 wherein the recombinant organism transformed with a vector that codes for the expression of Thermus aquaticus DNA polymerase is E. coli.

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